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The Effect of S-triazines and Nitrobenzene on the Degradation of Toluene and Napthalene in Solid Phase Systems

Samandra Demons

THE EFFECT OF S-TRIAZINES AND NITROBENZENE ON THE DEGRADATION OF TOLUENE AND NAPTHALENE IN SOLID PHASE SYSTEMS

by

SAMANDRA TASHAUN DEMONS

Under the Direction of George E. Pierce

ABSTRACT

Nitrogen is known to be a limiting factor in polluted environments, however many studies overlook the potential role for nitrogen to significantly influence the removal rate and efficiency with which microorganisms can degrade aromatic hydrocarbons. In this study, inoculated and uninoculated aerated soil microcosms containing different s-triazines were examined for their ability to efficiently and rapidly treat contaminated soils containing naphthalene, nitrobenzene, and toluene (NNT), via a microbial consortium consisting of *Pseudomonas*, *Rhodococcus,* and *Aeromonas*. After an experimental period of 14 days, greater than 90% degradation of NNT supplemented with different s-triazines, at concentrations of 1000-3000 ppm was observed. A difference in the degradation of NNT was seen in inoculated box reactors supplemented with cyanuric acid, melamine, and atrazine in comparison to uninoculated box reactors. Combined usage of 16s rDNA and 16s rRNA analysis was then applied to study the bacterial communities, and determine the abundance and survival of inoculated strains within box reactors contaminated with NNT. The bacterial diversity within clone libraries obtained illustrated a dominance of *proteobacteria* and gram positive bacteria. Analysis from clone libraries also showed that inoculated strains did survive within each condition, but were not the most predominant members present in the communities. This research shows that significant removal of NNT can

be achieved in two weeks with the supplementation of one of the s-triazines. However, differences in degradation and the microbial populations present within contaminated communities will be seen depending on which nitrogen sources are used and whether or not environments are bioaugmented or not.

INDEX WORDS: Bioremediation, Solid Phase Systems, Toluene, Naphthalene, Nitrobenzene, Community Analysis

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DEDICATION

To my parents Sandra and Samuel Demons

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I. INTRODUCTION

The world is dependent on oil. The total world consumption of petroleum was over 13.1 billion liters per day in 2003 (US EPA, 2006). Oil spills are inevitable because of the high consumption of petroleum products used today and through natural sources. Oil contamination typically through human activities, originate from petroleum refining and transport activities, through the discharge of industrial effluents or from accidental release, and from industries related to the transformation of fossil fuels and derived products (Wilson and Jones, 1993). During the past 20 years bioremediation has gained in acceptance as an alternative technology for oil pollutant removal. Bioremediation has been defined as the process of enhancing microbial activity to remove pollutants from soil and water (Atlas and Pramer, 1990) due to complex molecules being transformed to carbon dioxide and water (Goldstein *et al.*, 1985). However, successful bioremediation involves other factors including economics, public perception, and comparison with alternative technologies (Wick and Pierce, 1990).

Bioremediation includes methods that reduce mobility and migration of the contaminants, preventing their spread to uncontaminated areas, thus reducing the risk that contaminants pose to the environment. For the most part, remediation of near surface contamination relies on excavating the soil and treating excavated soil in separate areas or treatment facilities, e.g. thermal treatment and landfilling. Incineration, although costly, is a very effective treatment method, but after incineration the soil has lost most of its nutritional value and structure. Landfilling does remove the contaminants from the affected site but relocates the problem to another site. Landfilling also may incur increased costs through dumping and transporting contaminated soils into constructed landfills (Lageman *et al*., 2005). Therefore, it is evident that

new innovative treatment technologies, such as solid-phase bioremediation exist to address contaminated soils.

Solid-phase bioremediation is a process that treats soils in above-ground treatment areas equipped with collection systems to prevent any contaminant from escaping the treatment. The contaminated material is placed in an enclosure over an air distribution system where aeration is provided by pulling air throughout the material with an air pump. Volatile compounds are controlled in this type of system since they are part of the air stream that is drawn through the pile and may be captured volatile organic traps. Moisture, nutrients, and oxygen are also controlled to enhance biodegradation of the treatment area.

Microorganisms have the ability to utilize hydrocarbons as sole sources of energy and carbon (Zobell, 1946). Hydrocarbons provide an abundant source of carbon and energy to naturally occurring bacteria, which results in the complete degradation of the complex molecules, potentially leaving harmless carbon dioxide and water (Goldstein *et al.*, 1985). Many aromatic hydrocarbons, such as toluene, naphthalene, and benzene, are made naturally by plants (Hamamura *et al*., 2007); and are produced during burning of vegetation in forest and bush fires, by volcanic activity, and by plant and bacterial reactions (Wilson and Jones, 1993). Microorganisms have been exposed to these natural products for millions of years and have evolved enzymes necessary to obtain carbon and energy from them. Utilization of aromatic hydrocarbons is dependant upon both environmental conditions and the specific chemical nature of the compound itself (Zobell, 1946; Atlas, 1981).

Many researchers have defined bioremediation based on the potential of microorganisms to remove specific carbon sources. Nitrogen is known to be a limiting factor in polluted environments. However, many studies overlook the potential role for nitrogen to significantly

influence the removal rate and efficiency with which microorganisms can degrade hydrocarbons. Nitrogen is required for the synthesis of proteins. Aromatic hydrocarbon degraders have been found in many diverse environments because hydrocarbons and similar compounds are naturally occurring in many environments. It is hypothesized that different nitrogen sources, such as nitroaromatics and heterocyclic compounds such as the s-Triazines, can serve to induce genes in microorganisms that will permit a diverse number of hydrocarbons to be degraded and for this activity to be sustained in biologically based remediation technologies. Upon induction, the microorganisms will then utilize contaminants as substrates for energy, resulting in production of cellular biomass, metabolites, and CO_2.

Aromatic Degrading Microbial Community

When aromatic hydrocarbons are exposed to soil environments and during the subsequent degradation of these aromatic hydrocarbons, shifts in microbial populations occur (MacNaughton *et al.*, 1999). The number of culturable heterotrophs in environmental samples does not change significantly following an oil release to soil. Although, specific groups such as aromatic hydrocarbon degraders may be changed, with their numbers increasing from 1% up to 10% of the population (Atlas *et al*., 1991). Duncan *et al.* (1997) showed that the total number of heterotrophs in contaminated and uncontaminated soils was similar, though the numbers of aromatic hydrocarbon degraders and sulfate-reducing bacteria were higher in environments exposed to oil. Similarly, in a study by MacNaughton *et al.* (1999), the number of aromatic hydrocarbon degraders at an oil amended site initially increased, but slowly declined after 14 weeks.

A number of studies have reported a decrease in microbial diversity after aromatic hydrocarbon exposure (Atlas *et al.*, 1991). The use of toluene, naphthalene, and nitrobenzene as carbon sources will enrich only certain community members. Lindstrom *et al.* (1999) demonstrated a decrease in diversity in oiled soils by showing that remaining microbial populations consisted mostly of metabolic generalist and aromatic hydrocarbon-degrading organisms. These results cited above are not limited to natural environment since, even in closed systems such as bioreactors, enrichment of some community members due to the presence of aromatic hydrocarbons may result in an apparent rather than an actual decreases in diversity (Stoffels *et al.*, 1998; Greene *et al.*, 2002).

The distribution of aromatic hydrocarbon utilizing microorganisms should reflect the historical exposure of the environment to aromatic hydrocarbons. Many laboratory studies have demonstrated increases in populations of hydrocarbon utilizing microorganisms when environmental samples are constantly exposed to petroleum hydrocarbons. Cerniglia (1992) showed that chronic exposure did not necessarily increase heterotrophic microorganisms, but selectively increased hydrocarbon degrading populations**.**

There was a theory that impacted sites actually had increased diversity, and that lower diversity was a sign of a stable ecosystem (Cook and Hutter, 1981). In a stable, unstressed ecosystem, inoculated organisms will probably be out competed by native species since there is no significant stress that would select for them and ensure their survival. Therefore, it is easier to introduce microorganisms in a highly diversified, unstable environment since there is less of a chance of competitive inhibition by native microorganisms.

Degradation of Aromatic Hydrocarbons

Heitkamp *et al.* (1987) examined naphthalene degradation in microcosms containing sediments from environments that had prior exposure to aromatic hydrocarbons, pesticides and herbicides. Results showed that naphthalene could be degraded to CO_2 in microcosms by the native organisms. However, the rate of mineralization differed depending on the environments where samples were taken. The hydrocarbon contaminated environment showed the greatest mineralization rates followed by those exposed to pesticides and herbicides, and those found within pristine environments.

Nature of Compounds

Due to the hydrophobic nature of polycyclic aromatic hydrocarbons (PAHs), most of the compounds present are preferentially partitioned to soils/sediment with organic matter, and increasingly so with higher ring structures (Leahy and Colwell, 1990). Higher rates of degradation of hydrophobic compounds may occur with sediment present due to the degradation of sorbed compounds by attached bacteria (Guerin and Boyd, 1992). It also has been suggested that the ability to access sorbed substrate by attached species was species specific or was facilitated in selected species. Poeton *et al.* (1999) saw that hydrocarbons sorbed to soil were degraded by a *Pseudomonas* that was chemotactic and attached to soil particles reversibly.

Cell Surface Carrier

Woodchips are normally used as a bulking agent; however they also can serve as a surface for attachment of the bacterial inoculum. The pieces are light yet resistant to compaction, allowing for unrestricted air flow so as to prevent anaerobic zones. The organic

contents of the woodchips tend to bind the organic contaminants strongly, making efficient recovery of the contaminants by water washing difficult, protecting nearby bodies of water from the effects of run-off (Johnsen *et al.*, 2004). If the woodchips in a biopile were placed on a concrete pad with a water collection system, the contaminant tainted leachate would simply be recycled through the system to remove any residual contaminants.

There are potential nutrient sources or growth factors in woodchips in the form of humic substances. Fan and Scow (1993) stated evidence suggesting that humic substance can stimulate microbial metabolism. However, a study done by Harms and Zehnder (1995) implies that a surface area for attachment rather than humic substance enhanced substrate utilization.

Addition of Inocula to Stimulate Bioremediation

In order to achieve the highest rate of degradation of any contaminant there must be a sufficient amount of specific degraders present in the environment from which removal is needed. Most non-impacted communities consist of less than 1% of hydrocarbon degraders (Hamme, et. al 2003). To rapidly and effectively remove aromatic hydrocarbon contamination the process of seeding the environment with known degraders is often used. Dibble and Bartha (1979) reported that the initial indigenous population of oily-sludge-degrading bacteria was between 10^3 to 10^4 CFU/g of soil. Anything less than 10^5 CFU/g of soil would not be capable of removing the majority of the contaminant resulting in poor bioremediation results and/or bioremediation at a severely reduced rate. After the addition of the bacterial consortium, they saw higher rates of aromatic hydrocarbon degradation. Mishra *et al.* (2001) evaluated the addition of an inoculum to stimulate bioremediation of contaminated soils where the indigenous population of aromatic hydrocarbon-degrading bacteria in the soil was very low. They found

that the application of a bacterial consortium and nutrients resulted in high biodegradation yields (94.7% removal of aromatic fraction) as opposed to controls that were not inoculated which resulted in only 14% removal.

Microbial Community Analysis

Microbial diversity in soil may be large. One challenge of any bioremediation project is that of identifying the populations and microorganisms which have key roles in the degradation of aromatic and PAHs. Only 12% of soil microbial populations are culturable and these cultivable microorganisms may not be the important degraders within a community. One way to gain insight into bacteria that are actively being involved in degradation process is by exploiting culture-independent methods that permit examination of the diversity and composition of the metabolically active member present in highly polluted soils with aromatics (Nogales, 1999).

The major players in the degradation process can be evaluated through community analysis so as to not overlook any genera that may be poorly characterized due to unculturability. The use of 16S rDNA or rRNA is one of the most common approaches for community analysis since results obtained from 16S rDNA indicate which species are present in the environment and results from rRNA indicate which species are truly active. Smit *et al.*(2001) used 10 samples (10g each) from each soil type studied, so as to obtain and accurate representation of microorganisms in the soil environment. Once DNA is isolated, PAH-degrading bacteria can be characterized from the contaminated soil by looking at the partial 16S-rDNA sequences. All strains are then determined by using Basic Local Alignment and Search Tool (BLAST) similarity searching and by performing biochemical test (Eriksson *et al.*, 2001).

Isolation of Aromatic Hydrocarbon Degrading Bacteria

Enumeration of potential oil-degrading bacteria by their isolation on specific media has become a benchmark in many bioremediation studies, although many bacteria within the natural environment are dormant or unculturable on the media used (Toro *et al.*, 2006)**.** Therefore it is essential to show by combined chemical and microbiological methods that the oil-degrading bacteria are truly active.

Communities found in sludge normally consist of robust microorganisms that might succeed in colonizing contaminated soils. Sources such as this have been poorly applied in soil bioremediation (Dejonghe *et al.*, 2001; Juteau *et al.*, 2003; and Nano *et al.*, 2003). Toro *et al.* (2006) used a consortium of non adapted microorganisms from compost material to study aerobic bioremediation of PCB –contaminated soil. They found that the constructed consortium increased the cultivable PCB degraders in the solid phase reactors and enhanced the biodegradation of PCB from 50% to 100%. Thouand *et al.* (1999) showed that biodegradation capabilities of bacteria isolated from activated sludge, after being adapted to oil components, were equivalent or superior to commercial bacterial formulation sold as soil amendments for bioremediation processes.

Analysis of Substrate Interactions during Biodegradation

Substrate interactions are important in the degradation process. Contaminated soils usually contain more than one compound. Therefore, it is imperative that organisms augmented or indigenous to that contaminated environment posses machinery necessary to degrade multiple compounds collectively. Benzene and toluene are often found together in contaminated soil and groundwater. *Pseudonmas* sp. 55595 is a wild type organism that is capable of simultaneously

mineralizing both compounds. This organism does not recognize benzene as a substrate and therefore, requires toluene to act as an inducer for benzene degradation. Enzymes that catabolize toluene are the same enzymes that degrade benzene (Collins and Daugulis, 1991). The presence of toluene enhanced the degradation of benzene probably because benzene was degraded by enzymes induced by toluene. Bouchez *et al.* (1995) saw that in mixtures of two individually degradable PAHs, either preferential degradation of one PAH or reduced degradation rates of both PAHs were observed, indicating metabolic competition.

Chemotaxis toward Aromatic Hydrocarbons

Most bioremediation systems in operation today rely on microorganisms native to the contaminated sites. These systems are limited by the capabilities of the native microbes. Augmentations of contaminated sites with known aromatic and PAH degraders may aid in the degradation processes.

With regards to augmentation of a contaminated site, organisms inoculated in various soils should have some form of taxis. Chemotaxis is a generalized term describing the process by which cells respond to environmental conditions by moving towards favorable environmental conditions and away from unfavorable ones. A variety of environmental cues can elicit a taxis response in bacterial cells including, but not limited to chemicals, pH, light, oxygen, reduction potential, magnetic field, temperature, osmolarity (Atlas, 1981). Bacteria swim toward or away from a chemical stimulant in a guided, nonrandom manner. Potential degrading microorganisms in the environment must be in close proximity to the organic compounds of interest before problems of solubility, concentration or transport can be addressed. In a soil environment contaminated with aromatic hydrocarbons the pollutants are not homogeneously spread

throughout the treatment area. Therefore, it is essential that either the bacteria have some form of chemotaxis to the compounds that will allow movement towards higher concentrations of substrates that may be adsorbed to soil particles, or traveling within a plume (Singh and Ward, 2004). The alternative would be for soils to be mixed periodically (costly). Chemotaxis toward aromatic and PAHs such as toluene and naphthalene have been observed (Pandey and Jain, 2002). The discovery of the chemoreceptors nah-Y naphthalene in *Pseudomonas putida* G7 and tfdK in *Ralstontia eutropha* JMP13ch4 and the requirement of this receptor for active uptake have shown that chemotaxis, and biodegradation are linked (Parales and Harwood, 2002).

Optimal Environmental Conditions for Aromatic Degradation

Microorganisms in the soil can only biodegrade aromatic and PAHs within a limited range of favorable conditions. Appropriate environmental conditions must exist for microorganisms to sustain life. These conditions include appropriate pH, temperature, oxygen, nutrients, water, and lack of inhibiting or toxic compounds.

The efficiency of bioremediation in soils is the best when the pH is near 7 (Dibble and Bartha, 1979). Most soils are very acidic and require the addition of some neutralizer such as dolomitic lime which can only go to a pH of 8.5. Heterotrophic bacteria and specific aromatic hydrocarbon degraders favor pHs near neutrality (Atlas, 1990). Along with pH, temperature also plays an important role in the removal of hydrocarbons. Not only does temperature affect the physical nature of the compound, but also determines the microbial community that will degrade a particular compound and the rate at which hydrocarbons are metabolized. Hydrocarbon degradation does occur at lower temperatures but the highest rates of degradation for aromatics usually occur in the range of 30^{o}- 40^{o}C (Atlas and Pramer, 1990).

Hydrocarbon contaminated soil environments contain aerobic and anaerobic zones. Molecular oxygen is required for high rates of aromatic hydrocarbon degradation since the initial step in the aerobic breakdown of aromatic and PAHs involves the enzymatic oxidation of these compounds by oxygenases (Leahy and Colwell, 1990). It has been observed that hydrocarbon biodegradation slows down when oxygen concentration drops below 2-5% (Hurst *et al*., 1997)**.** Therefore, it is necessary to provide soil systems with sufficient aeration, so that aerobically degrading organisms can metabolize aromatic hydrocarbons completely.

The application of nutrients, in the form of fertilizer, to enhance the microbial degradation of PAHs is a commonly used practice (Johnson and Scow, 1999). Crude oil degradation was increased with the use of Inipol EAP22, an oleophilic fertilizer, in the Exxon Valdes spill in Alaska's Prince William Sound. CO_2 measurements showed significant removal of compounds shortly after the addition of the fertilizer. The effects of the application of phosphorous and nitrogen were studied for the bioremediation of contaminated soil and results indicate that these variables correlated with PAH biodegradation (Leys *et al*. 2005).

Organisms require water to survive. Not only does it aid in movement of microorganisms, but also in diffusion of nutrients and biochemical processes that may occur within an organism. The optimum moisture content for stimulating hydrocarbon biodegradation ranges from 50 to 80%. If the soil is to dry, bacterial growth and metabolisms will be greatly reduced or even inhibited (Singh and Ward, 2004). However, if the soil is too wet, soil environments become anaerobic which are not that conducive for the degradation of most hydrocarbons.

Rationale

Naphthalene, nitrobenzene and toluene (NNT) are environmental pollutants suspected of being carcinogenic; with naphthalene being the most persistent in the environment. Cost effective, reliable, and quick methods to remove high concentrations of these pollutants are needed. Many studies have focused on the degradation of these pollutants either as a single contaminant or in sterile environments, which is unlikely in real world environments. Nitrogen is often a limiting factor in bioremediation studies. It is imperative that the bioremediation system developed use inexpensive nitrogen sources, which can be released slowly into the environment to ensure consistent levels of degradation. Solid phase systems, which efficiently degrade high concentrations of NNT supplemented with slow release nitrogen sources, in real world settings, can be used as prototypes to validate the use of implementing a full scale remediation project.

Ultimately, it is the intent to develop a low-cost method to treat soils with high concentrations of aromatics and PAHs. The goal of this study is to stimulate the activity of native or re-introduced native microorganisms to enhance biodegradation of aromatic hydrocarbons, through the adjustment of parameters such as oxygen flow, pH, moisture content, and nutrient addition. In addition to that, major genera involved in the degradation process will be identified, and microorganisms that degrade high concentrations of NNT supplemented with one of the s-Triaizines will be isolated and identified.

II. MATERIALS AND METHODS

Microorganisms

DAP strains 66, 119, and 626 were obtained from the American Type Culture Collection (ATCC, Vienna, VA). These strains were all isolated from soil samples taken from Bridgewater, NJ (Pierce and Smith, 1997) and have been used in the degradation of aromatic compounds found in PAH waste materials. Each strain was revived and sub-cultured using nutrient agar and/or nutrient broth and incubated at 30^{o}C. Initial acclimation of cultures to hydrocarbons was done using nutrient broth supplemented with 200 ppm each of toluene and naphthalene (NT) and 25ppm of nitrobenzene (NoBz). Cultures were then transferred to Stanier's minimal media (Stanier, 1966) supplemented with 200 ppm each of NT, and 50 ppm of NoBz, and incubated at 30^{o}C. As the cultures grew, they were successively transferred to Stanier's media with increasing concentrations (100-1000ppm) of aromatic hydrocarbons. All cultures were maintained on Stanier's media supplemented with 200 ppm each of NT, and 50 ppm of NoBz at 4^{o}C for up to three months.

Preparation of Media

The required nutrients for the growth of DAP strains 66, 119, and 626 inoculated into a reactor, was provided by a Stanier's mineral medium with the following composition per 1L of solution : 2.5mg EDTA, 10.95mg $ZnSO_4.7H_2O$, 5mg $FeSO_4.7H_2O$, 1.54mg $MnSO_4.H_2O$, 0.392mg $CuSO_4.5H_2O$, 0.248mg $Co(NO_3)_2.6H_2O$, 0.177mg $Na_2B_4O_7.10H_2O$, 289mg $MgSO_4$, 66.7mg $CaCl_2.2H_2O$, 0.185mg $(NH_4)_2Mo_7O_{24}.4H_2O$, 1.98mg $FeSO_4.7H_2O$, 200mg nitriltroacetic acid, 146mg KOH, 3984mg Na_2HPO_4, 1828mg NaH_2PO_4. Various amounts of

NNT, cyanuric acid, melamine, and atrazine were aseptically added to Stanier's medium, to give concentrations between 25-150 ppm for nitrogen sources and 500-1000ppm for carbon sources.

Chemicals used for Carbon and Nitrogen Sources

All chemicals were purchased from Sigma Aldrich to include toluene, naphthalene, nitrobenzene, atriazine, melamine, and cyanuric acid.

Experimental Design of Box Reactors

Degradation experiments were performed in six box reactors with dimensions of 10"X10"X10" (see figure 1). Reactors were composed of 3/8 inch plexiglass, lined with teflon sheeting, and fitted with ¼ inch stainless steel Swagelok fittings. Woodchips (Pine nuggets) were pretreated with dolomitic lime and inoculated with 10^6 cells before being placed in reactors. Ambient air was drawn through the reactors to deliver air to contaminated material. Perforated tubing was placed under the contaminated material, and air was pulled throughout system through two granulated activated carbon (F300 GAC) traps (used to collect volatiles), a drierite trap, a mass flow meter, and a vacuum pump. The air flow rate was maintained at 300ml/min, so as to not strip away moisture from woodchips. Nutrients were supplied weekly with the use of a syringe, to ensure even dispersal, and the moisture content and pH were maintained within each box reactor.

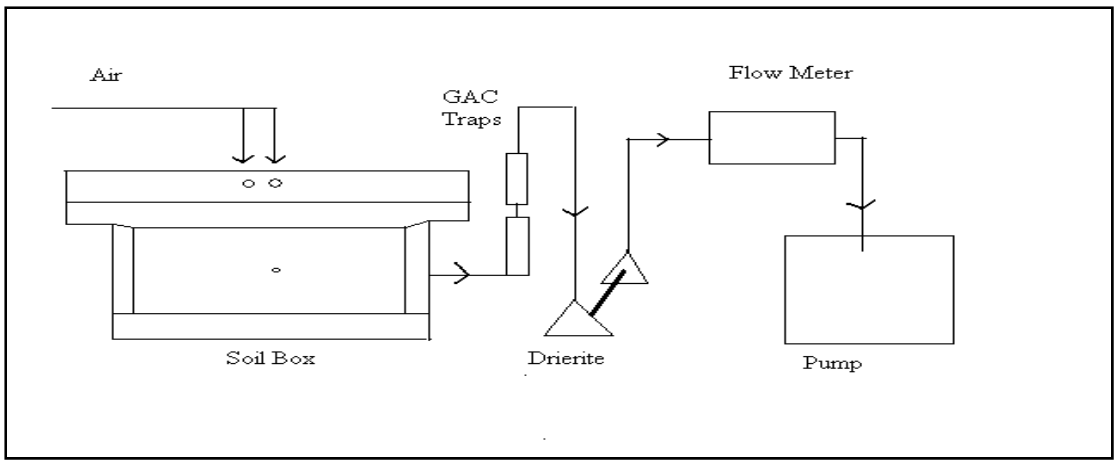

Figure 1. Schematic representation box reactor.

Preparation Inoculum in Box Reactors

The soil box inoculum was prepared by inoculating hydrocarbon acclimated DAP strains 66, 119 and 622 into separate flasks Stanier's broth containing 500 ppm each of NT, and 100 ppm of NoBz and incubating them in a rotary shaker for 1 week at room temperature. The cells were then harvested by centrifugation and washed twice with phosphate buffer and re-suspended in minimal broth before being placed in a soil box. The concentrated cells were combined to form a mixed community that was re-circulated on woodchips in a column until all the liquid mixture was absorbed. Once the woodchips are inoculated, sterile dolomitic lime was added in order to keep the pH neutral.

pH Control in Box Reactors

The pH of a woodchip leachate was checked by adding equal amounts of water to equal amounts of woodchips, mixing that sample, and determining the ph. Frequency of sampling required that minimal samples were taken for pH checks. Each sample (2 g of chopped woodchips) was mixed with 2 ml of water and then sonicated for 1 min, allowed to stand for 5

minutes (settling of particulates), before measuring the pH. Dolomitic lime was added to the sample. The amount of lime required to neutralize box reactors was calculated from the amount of lime added to neutralize sub-samples.

Monitoring of Biomass in Box Reactors

Biomass was monitored by total viable cell counts using 2 types of media: R2A (BD, Sparks, MD) for heterotrophs, and Stanier's supplemented with 50 ppm of either cyanuric acid, melamine, or atrazine, 500ppm of NT, and 50 ppm of NoBz for specific carbon degraders. Random samples were periodically taken from the box reactors, serially diluted in 50mM sterile PBS (pH 7.0), and streaked onto plates. All plates were incubated at 30^{o}C. R2A plates were counted after 48h, and Stanier's plates supplemented with one of the nitrogen sources along with NNT were counted after 5 days.

Analysis of Aromatic Hydrocarbons

Liquid sample extractions were analyzed using gas chromatography (HP5890, series II, Palo Alto, CA) equipped with a fused silica megabore column (75m in length, 0.53mm i.d., 3um film; DB-624, Agilent, J&W, Palo Alto, CA) and a flame ionization detector. Helium was used as the carrier gas at a flow rate of 18ml/min. The temperature for the column, injector and detector were 300 ^{o}C, 300^{o}C, and 250^{o}C, respectively. A liquid tight microsyringe (Agilent 5181-1273) was used to inject 0.5µl of sample into GC. GC for all chemicals was run as a temperature gradient. The detection limits for toluene, naphthalene, and nitrobenzene were 5ppm, 10ppm, and 3ppm respectively.

Volatilization

Volatile organic compounds (VOCs) from each box system were trapped in the 2 VOC traps containing GAC. VOC traps were prepared by first washing the empty trap with toluene, and then dried for 10 min using N_2 gas. Next, 3.5 g of F300 GAC was added inside steel tubes, closed from both ends with the use of glass wool, and attached to the soil box using Per Fluoro Alkoxy (PFA) tubing. To measure the rate and amount of compound lost to volatilization all VOCs were solvent extracted from the GAC with methylene chloride and methanol (85:15) and analyzed on the gas chromatograph (GC) (CYTEC protocol).

Extraction of VOCs from Woodchips

One gram of woodchips was placed in a 40 ml crimp top vial containing 5 ml of methanol and 33 ml of methylene chloride and allowed to shake for 24 hours at 30 rpm. Samples were taken from the vial using a gas tight syringe and analyzed on GC to determine residual compounds.

Chemotaxis

LB plates and Stanier's plates containing 0.3% agar were poured. Stanier's plates contained 25 ppm of cyanuric acid, melamine or atrazine, 500 ppm each of toluene and naphthalene, and 50 ppm of nitrobenzene. Ten hydrocarbon degraders isolated from activated sludge and woodchip (pre-exposed to NNT) were stabbed onto the center of the plates with sterile tooth picks. Plates were incubated in the 30^{o}C incubator for 5 days after which bacterial consumption of nutrients was measured by the diameter of microbial growth (Lanfranconi, *et al.* 2003).

Stanier's Test for Ring Cleavage

All cultures were grown on Stanier's slants supplemented with 0.1% Sodium p-hydroxybenzoic acid and grown on R2A slants. Growth from agar was scraped off and suspended in 2mls of 0.02M Tris Buffer, pH 8.0. Tubes were shaken with 0.5 ml of toluene and 3.5 mg/tube of 3,4-dihydroxybenzoic acid was added. A yellow color after this step indicates meta-cleavage. If no yellow color developed the tubes were shaken for 1 hour at 30^{o}C. 1.0 gram of (NH4)2SO4 was added along with 1 drop of 1% Sodium Nitroprusside. Ammonia (0.5ml) was then added to the solution and the development of a purple color indicated ortho-cleavage. *P. putida* mt-2 (positive for meta-cleavage) and *P. oleovorans* (positive for ortho-cleavage) were used as positive controls (Stanier *et al*., 1966).

Metabolism of NNT Supplemented with Cyanuric acid, Melamine, or Atrazine

A mixed culture containing DAP strains 66, 119, and 626 along with isolates obtained from box reactors and activated sludge was tested for their ability to metabolize NNT supplemented with either cyanuric acid ,or melamine, or atrazine. All organisms were grown on Stanier's plates containing concentrations of 500-1000ppm each of NT, 50-150ppm of NoBz, and 50-100ppm of cyanuric acid, melamine, or atrazine and incubated for one week at 30^{o}C. Growth was checked and scored as either excellent, very good, good, little, marginal, and non-detectable growth.

Extraction of Genomic DNA from Pure Cultures

Isolated bacteria were cultivated in 5 ml of nutrient broth at 30^{o}C with rotary shaking at 180 rpm. Two ml of cells were harvested by centrifugation for 5min at 13000 rpm at room temperature (RT, 25-27^{o}C). Three hundred microliters of sodium phosphate buffer (0.12 M, pH

8.0) was used to resuspend the cell pellet and 30 µl SDS-solution (10%) was added to sample. Samples were allowed to incubate at RT for 10 min, followed by the addition of 100mg of acid washed glass beads (0.1mm diameter). Sigma tubes were placed in a bead beater for 30 sec and processed two times at 2500 rpm. Samples were centrifuged (Beckman Microfuge Lite, Palo Alto, CA) at 13000 rpm for 10min at RT. After centrifugation, the supernatant was decanted to clean 2 ml microcentrifuge tubes and 300 µl of a chloroform:isoamyl alcohol (24:1) solution was added. The supernatant and chloroform:isoamyl alcohol were manually shaken for 1 min and centrifuged for 5 min at 13000 rpm (2X). The supernatant was transferred to a clean 2 ml tube. Sodium acetate was added at 1/10 the volume and the remaining space within the tube was filled with 100% ethanol, and vortexed. DNA precipitated for 1 hour at RT and then centrifuged for 15 min at 13000 rpm. The supernatant was discarded and 500µl of 70% ethanol was added to the DNA pellet and centrifuged for 15 min at 13000rpm. Ethanol was then discarded and the DNA pellet was dried using vacufuge (Eppendorff, Hamburg, Germany) for 5 min. The pellet was then resuspended in sterile water (Dr. Chin Lab, unpublished protocol). Extracted DNA was visualized by gel electrophoresis on a 1% agarose gel to obtain an estimate of the size and quality of the genomic DNA.

Extraction of DNA from Woodchips

DNA extraction from woodchip samples was performed using a Fast DNA Spin for Soil Kit ® as stated in manufacturer's protocol from Q-BIOgene (San Diego, CA).

Extraction of Total RNA from Woodchips

RNA extraction of woodchip samples was preformed as stated in manufacturer protocol for RNA Power Soil Kit® (MO BIO Laboratories Inc, Carlsbad, CA). After RNA was extracted, it was visualized by formaldehyde agarose gel electrophoresis.

Reverse Transcription PCR

cDNA was synthesized using Fermentas First Strand cDNA ® as stated in manufactures protocol (Hanover, Maryland). The 907R primer was used for reverse synthesis.

Primers

Six different primers were tested to amplify the 16s rRNA gene within the DNA samples. Aliquots of each primer were made in an effort to minimize contamination.

Table 1. Universal Bacterial Primers used to amplify 16s rRNA gene.

Primer Name	**Sequence (5'-3')**
16S-8F	AGAGTTTGATCMTGGCTCAG
16S-27F	AGAGTTTGATCMTGGCTCAG
16S-338F	ACTCCTACGGGAGGCAGC
16S-907R	GGTGACCTTGTTACGACTT
16S-1392R	ACGGGCGGTGTGTRC
16S-1492R	TACGGYTACCTTGTTACGACTT

PCR, Cloning, and Sequence Analysis

Each PCR reaction contained 11.75µl dH_2O (DNA-free), 5 µl Q solution (Qiagen), 2.5 µl 10X buffer, 2 µl DNTPs, 1µl 338 Forward primer, 1ul 907 Reverse primer, 1µl BSA 20x, 0.5 µl Temp and 0.25 µl Taq (Fermentas, Hanover, Maryland) , for a total volume of 25ul. The following thermocycler program was used: Step 1: 95^{o}C for 5 min (denaturation), Step 2 94^{o}C for 30 sec (denaturation), Step 3 51^{o}C for 30 sec (annealing), Step 4 72^{o}C for 1 min (extension), Step 5 72^{o}C for 10 min (final extension), and Step 6 4^{o}C hold time. Steps 2-4 were run for a total of 32 cycles. All PCR runs contained a positive and negative control in order to ensure that the PCR program was working properly and that no contamination was present. PCR products were visualized by gel electrophoresis. DNA samples were analyzed and considered positive if the expected 569 band amplicon was detected. Cloning of the 16s rRNA gene was achieved through the use of Topo Cloning Kit (Invitrogen, Carlsbad, California). After *E. coli* cells were transformed, they were plated on LB medium containing 100 µg/ml ampicillin and 100 µg/ml 5-Bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal). Positive clones were selected and inoculated in LB media containing ampicillin at 100 µg/ml, overnight at 37^{o}C. Plasmid DNA was extracted using Fast Plasmid Mini kit (Eppendorff). PCR analysis of plasmid DNA was performed to check for the correct insert size using the same thermocycler program and PCR reaction mix as previously described. Sequencing was done by Georgia State University core facility. All sequences were analyzed using Basic Local Alignment Search Tool (BLAST) to identify the organisms.

Enrichment and Identification of Hydrocarbon Degraders from Activated Sludge

One gram of activated sludge (Kindly provided by Atlanta Wastewater Treatment Plant) was sonicated in sterile PBS, serial diluted and plated on nutrient agar. Isolated colonies were acclimated, as previously described, and transferred to Stanier's media with increasing concentrations (100-1000ppm) of NNT. Identification using 16s rDNA analysis was completed on isolates able to degrade high levels of NNT.

III. RESULTS

Growth of DAP Strains 66, 119, and 662 on NNT Supplemented with either Cyanuric Acid, Melamine, or Atrazine

DAP strains 66, 119, and 662, previously induced to NNT, were evaluated on how well they grew individually or as a controlled mixed culture on different concentrations of NNT supplemented with cyanuric acid, melamine, or atrazine. Table 2 shows that while all individual strains grew well on increasing concentrations of NNT and cyanuric acid (NNT +C), the mixed culture consistently exhibited better growth at higher concentrations of NNT+C. There was a significant decrease in growth of strain 662 at the highest concentration of NNT +C, indicating that it had probably reached the concentration limit for NNT using cyanuric acid as a supplemental nitrogen source.

Table 3 shows that DAP strain 66 grew just as well as the mixed culture on higher concentrations NNT and melamine (NNT + M). Both DAP 66 and the mixed culture showed excellent growth on lower concentrations and good growth on higher concentrations of NNT +M. Strain 119 exhibited excellent growth on lower concentrations, but little to no growth was seen on higher concentrations of NNT+M. Similarly, 662 showed more growth on lower

concentrations rather than higher ones. In comparison to table 1, the organisms appear to prefer cyanuric acid as a supplemental nitrogen source over melamine when growing on higher concentrations of NNT.

Table 4 shows that strain 662 grew better on higher concentrations of NNT supplemented with atrazine (NNT+A) than DAP strains 66, and 119. These results are the reverse of the results obtained in the box reactor containing NNT+M. DAP strains 66 and 119 grew well on lower concentrations, but decreases in growth were seen at higher concentrations.

A noticeable change in growth was evident as concentrations increased using the various nitrogen sources. However, the mixed culture was the most consistent in exhibiting good growth on high concentrations of NNT supplemented with cyanuric acid, melamine, or atrazine. The scoring of the growth was based upon comparisons made within groups using the same nitrogen source. Based upon these results, it was determined that a mixed culture would probably degrade targeted compounds better than a pure culture using cyanuric acid, melamine, or atrazine as a supplemental nitrogen source.

Table 2. Growth of DAP strains 66, 119, 662, and a mixed culture consisting of all three strains on increasing concentrations of NNT+ C.

DAP Strain #	**NNT+ C(1)**	**NNT+C(2)**	**NNT+C(3)**	**NNT+ C(4)**
66	+ + +	+ + +	+ +	+ + +
119	+ + + +	+ + +	+ +	+ +
662	+ + +	+ + +	+ + +	+
Mixed Culture	+ + + +	+ + + +	+ + + +	+ + + +

NNT1= 25ppm of Cyanuric Acid , 250ppm each of NT and 50ppm of NoBz
NNT2= 50ppm of Cyanuric Acid , 500ppm each of NT and 100ppm of NoBz
NNT3= 75 ppm of Cyanuric Acid, 750ppm each of NT and 125ppm of NoBz
NNT4=100ppm of Cyanuric Acid, 1000 ppm each of NT and 150ppm of NoBz

-No detectable growth
+/- Marginal growth
+ Little growth
++ Good growth
+++ Very good growth
++++ Excellent growth

Table 3. Growth of DAP strains 66, 119, 662, and a mixed culture consisting of all three strains on increasing concentrations of NNT+M.

DAP Strain #	NNT+ M(1)	NNT+ M(2)	NNT+ M(3)	NNT+ M(4)
66	++++	++++	++	++
119	+++	++++	+	-
662	++	+	+	+/-
Mixed Culture	++++	+++	+++	++

NNT1= 25 ppm of Melamine ,250ppm each of NT and 50ppm of NoBz
NNT2= 50ppm of Melamine ,500ppm each of NT and 100ppm of NoBz
NNT3= 75 ppm of Melamine, 750ppm each of NT and 125ppm of NoBz
NNT4=100ppm of Melamine, 1000ppm of each and NT and 150ppm of NoBz

-No detectable growth
+/- Marginal growth
+ Little growth
++ Good growth
+++ Very good growth
++++ Excellent growth

Table 4. Growth of DAP strains 66, 119, 662, and a mixed culture consisting of all three strains on increasing concentrations of both NNT +A.

DAP Strain #	NNT+ A(1)	NNT2+ A(2)	NNT3+ A(3)	NNT4+ A(4)
66	++	+++	+	+/-
119	++	++	+	+
662	++++	++++	+++	++
Mixed Culture	+++	+++	++	++

NNT1= 25ppm of Atrazine, 250ppm of each NT and 50ppm of NoBz
NNT2= 50ppm of Atrazine, 500ppm of each NT and 100ppm of NoBz
NNT3= 75 ppm of Atrazine, 750ppm of each NT and 125ppm of NoBz
NNT4=100ppm of Atrazine, 1000 ppm of each NT and 150ppm of NoBz

-No detectable growth
+/- Marginal growth
+ Little growth
++ Good growth
+++ Very good growth
++++ Excellent growth

Degradation of 500ppm each of NT and 50ppm of NoBz Supplemented with either Cyanuric Acid, Melamine or Atrazine

Initially, all box reactors contained 500ppm each of NT and 50ppm of NoBz supplemented with cyanuric acid, or melamine, or atrazine as additional nitrogen sources. All boxes were run for a total of six weeks in order to adapt the microorganisms in the box reactors to environments containing NNT. The six week adaptation period included an initial feed followed by a refeed at the end of week three.

Figure 2 shows the degradation of 500ppm each of NT and 50ppm of NoBz in experimental and control boxes supplemented with cyanuric acid over the six week period. The rate of degradation, as shown by the slope of the line in table 5, shows that all control boxes had higher rates of degradation than experimental boxes before the refeed. However, after the refeed all of the experimental boxes had higher rates of degradation as opposed to control boxes. Volatilization of NNT is seen before and after the refeed. Figure 3 shows that more than half of the toluene within the experimental and control boxes, where cyanuric acid was the nitrogen source, was being volatized as compared to naphthalene and nitrobenzene that have less than 20% of their removal being attributed to volatilization. At the end of the initial feed and the reefed, NNT had been completely remediated from all boxes (figure 4).

Degradation of 500ppm each of NT and 50 ppm of NoBz supplemented with melamine in experimental and control boxes over a six week period of time is illustrated in figure 5. The rate of degradation, as is determined by the absolute value of the slope, indicates that nitrobenzene in the control box before and after the refeed was degraded faster than nitrobenzene in the experimental box (Table 6). Toluene in both the experimental and control boxes had very similar rates of degradation before and after the refeed. Naphthalene in the experimental and control boxes had similar rates of degradation before the refeed. However, the naphthalene control was degraded faster than naphthalene in the experimental box after the refeed. Figure 6 shows that toluene is the most volatile of the compounds being degraded. Greater than 50% of its removal is attributed to volatilization whereas less than 20% of naphthalene and nitrobenzene removal is due to volatilization. The percentage of NNT removal in figure 7 shows that only toluene was completely removed from boxes before the refeed and that after the refeed all of the compounds were remediated.

The degradation of 500ppm each of NT and 50 ppm of NoBz supplemented with atrazine over a six week period of time is shown in figure 8. Table 7 shows that the rate of degradation of nitrobenzene in the experimental box was faster than nitrobenzene in the control box before the refeed. After the refeed, the rate of nitrobenzene degradation in both experimental and control boxes was relatively the same. The degradation rate of toluene in the experimental box before and after the refeed was faster than in the control box. The rate of degradation of naphthalene in the experimental box was greater than in the control box before the refeed and only slightly greater after the refeed. Figure 9 shows that similar to the previous boxes supplemented with cyanuric acid and melamine, more than 50 % of toluene was removed by volatilization. Nitrobenzene in the control box, before the reefed, was volatized more than in boxes containing cyanuric acid and melamine as nitrogen sources. Naphthalene was volatized about the same as in boxes containing cyanuric acid and melamine. Figure 10 shows that before the refeed, only naphthalene in the control box remained in the box. However, after the refeed all compounds were remediated from the environment.

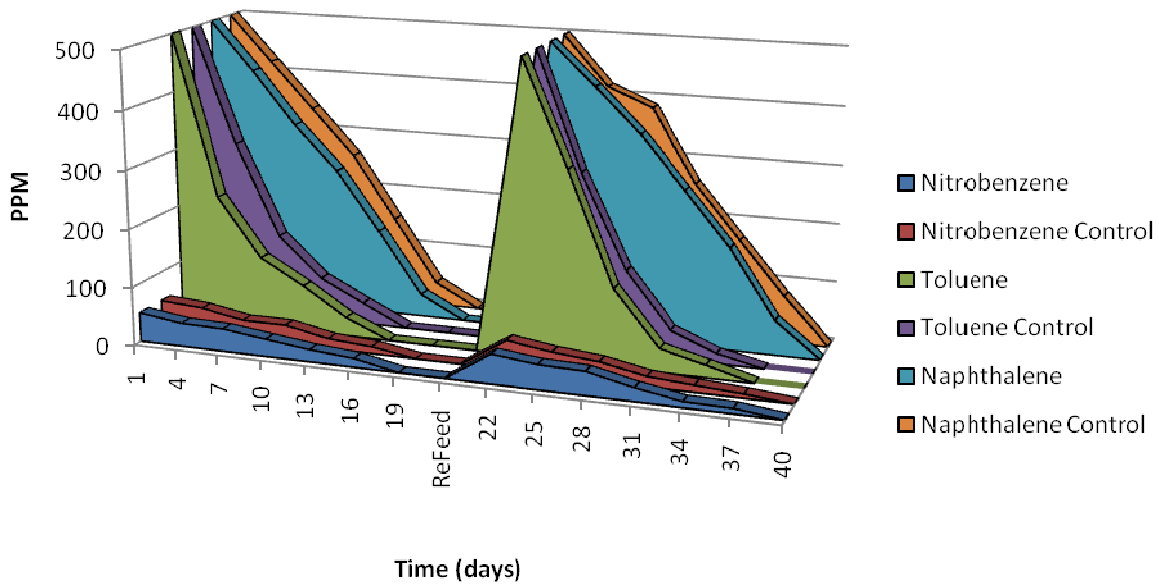

Figure 2. Degradation of 500ppm each of NT and 50ppm of NoBz, supplemented with cyanuric acid, over a 6 week period in box reactors.

Table 5. Degradation rate of 500 ppm each of NT and 50ppm of NoBz, supplemented with cyanuric acid, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Weeks 1-3	7.78	8.25	107.80	115.50	86.89	87.50
Weeks 4-6	8.78	8.35	124	121.80	86.32	85.25

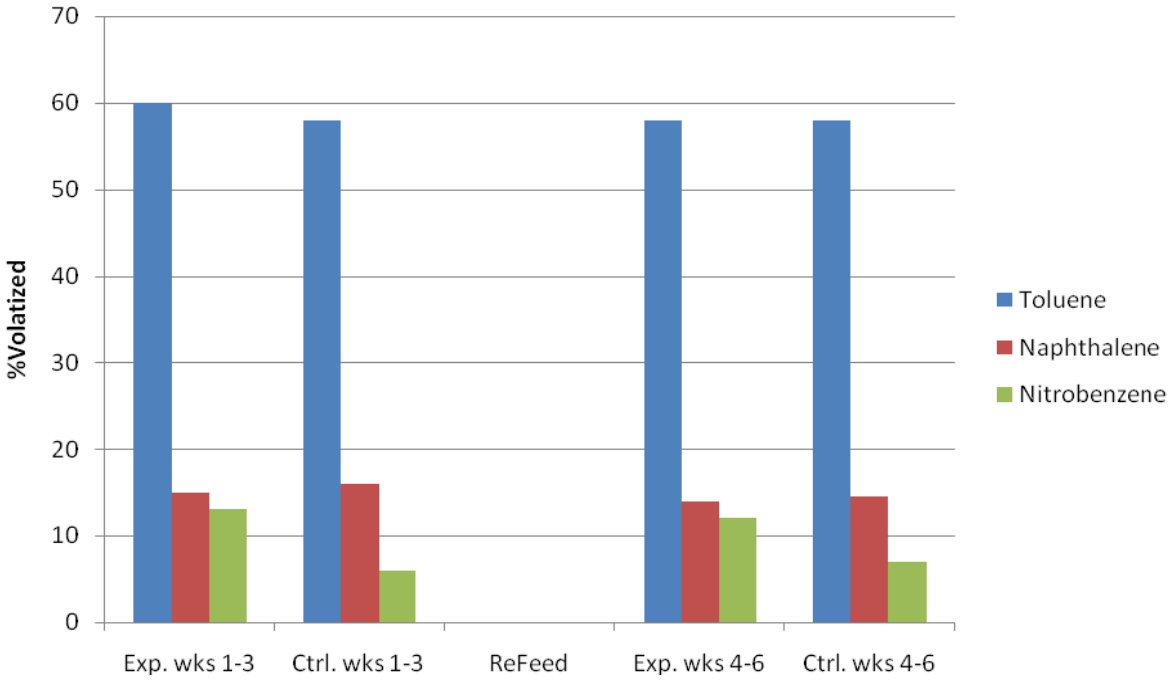

Figure 3. Percent loss of 500 ppm each of NT and 50ppm of NoBz, lost to volatilization in box reactors supplemented with cyanuric acid, as determined by VOC trap extractions.

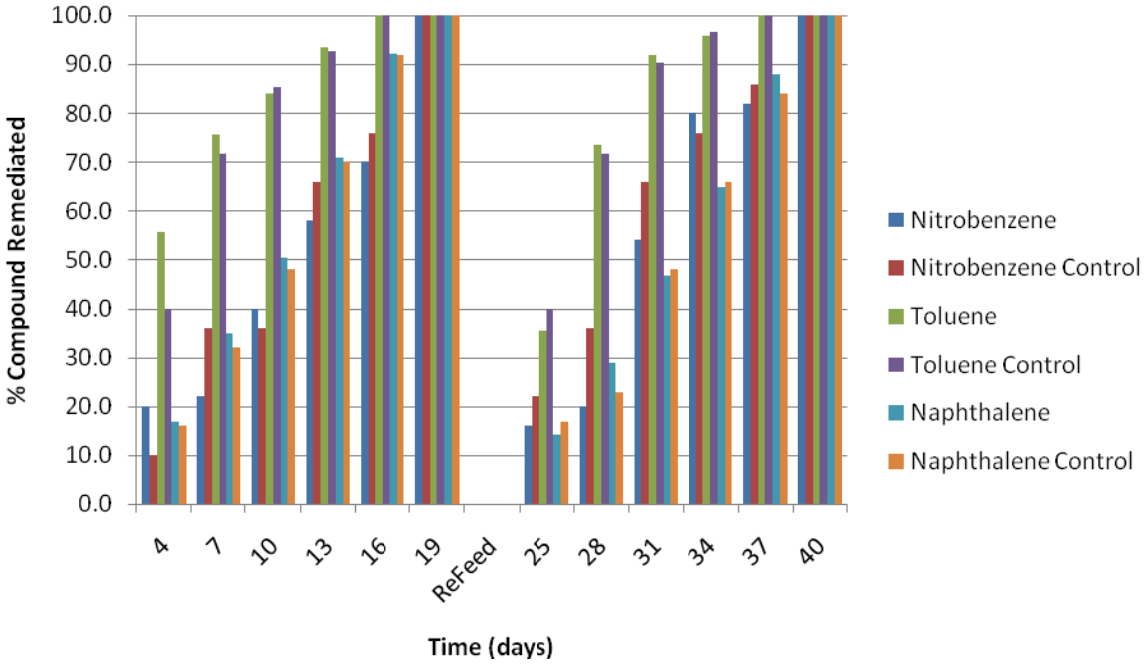

Figure 4. Percent remediated of 500ppm each of NT and 50ppm of NoBz, supplemented with cyanuric acid in box reactors.

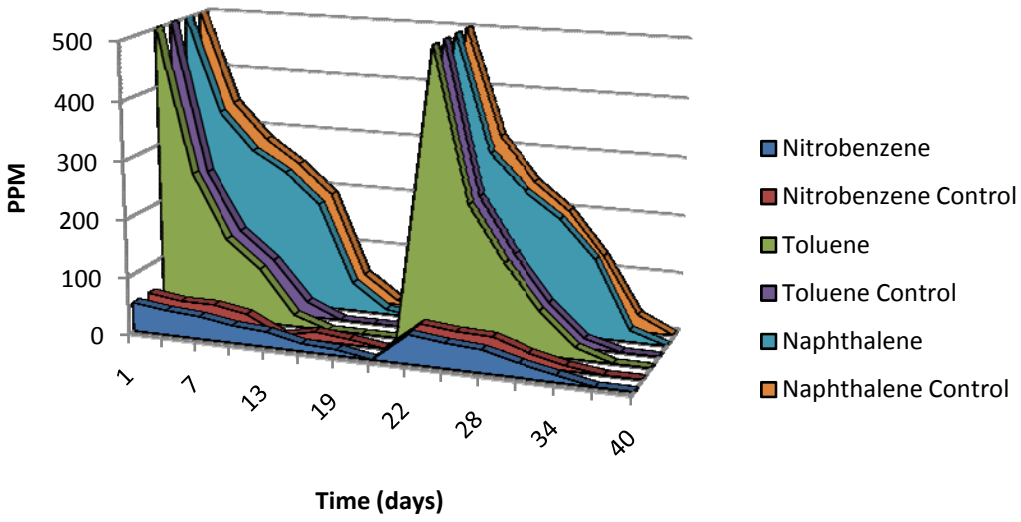

Figure 5. Degradation of 500ppm each of NT and 50ppm of NoBz, supplemented with melamine, over a 6 week period in box reactors.

Table 6. Degradation rate of 500ppm each of NT and 50ppm of NoBz, supplemented with melamine, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Week 1-3	6.89	7.75	111.70	109.50	76.21	76.96
Week 4-6	9.39	9.71	112.70	113.60	77.50	78.53

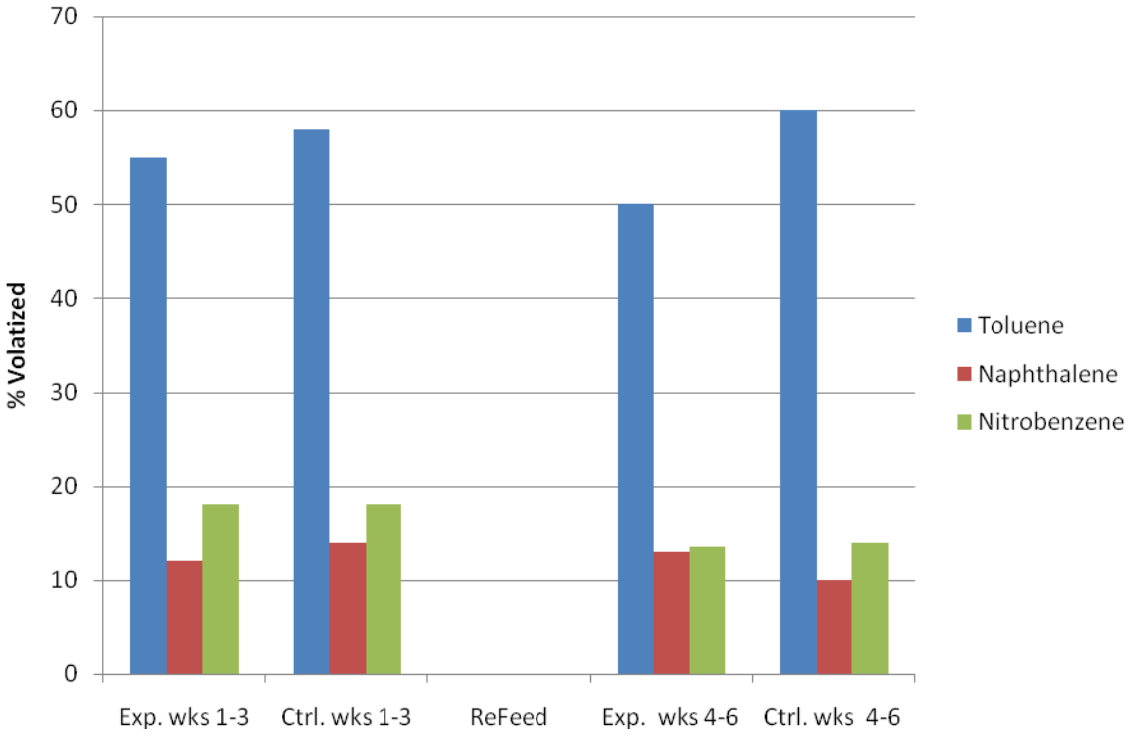

Figure 6. Percent loss of 500ppm each of NT and 50ppm of NoBz, lost to volatilization in box reactors supplemented with melamine, as determined by VOC trap extractions.

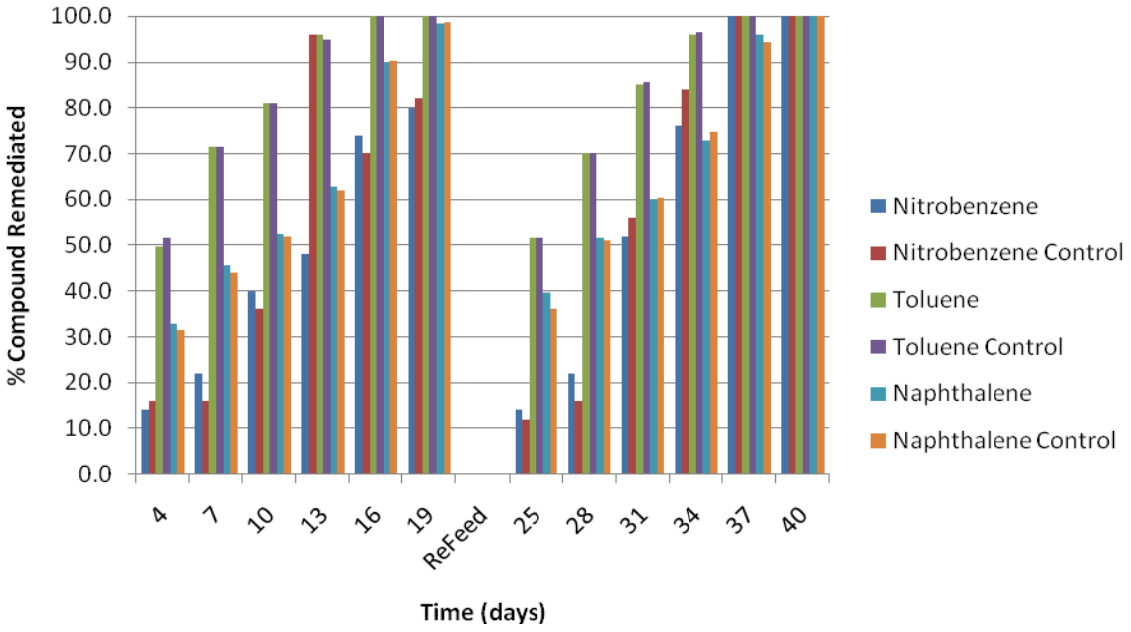

Figure 7. Percent remediated of 500ppm each of NT and 50ppm of NoBz, supplemented with melamine in box reactors.

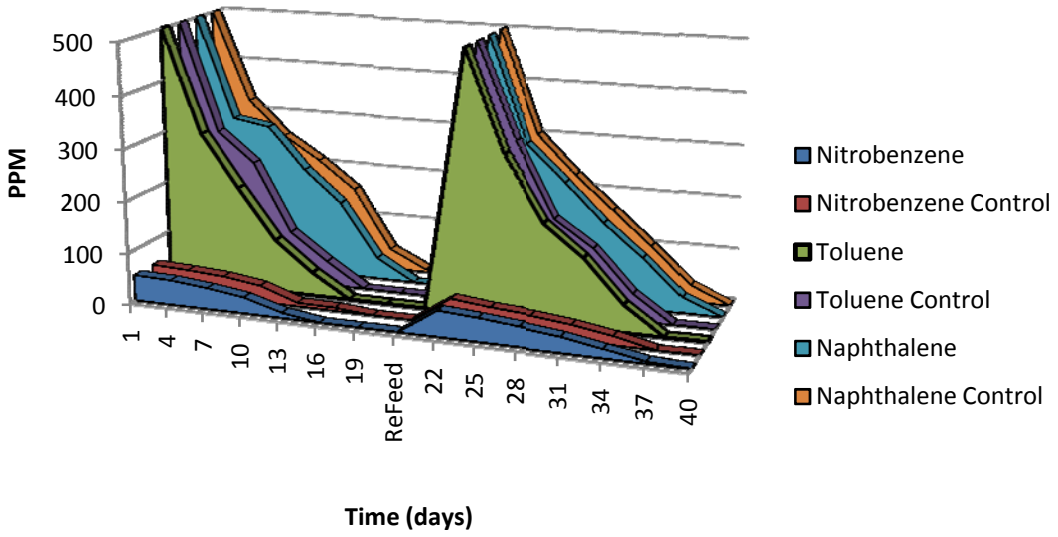

Figure 8. Degradation of 500ppm each of NT and 50ppm of NoBz, supplemented with atrazine, over a 6 week period in box reactors.

Table 7. Degradation rate of 500 ppm each of NT and 50ppm of NoBz, supplemented with atrazine, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Week 1-3	9.89	9.54	110.80	109.60	77.07	76.71
Week 4-6	9.04	9.07	108.40	107.80	77.46	77.36

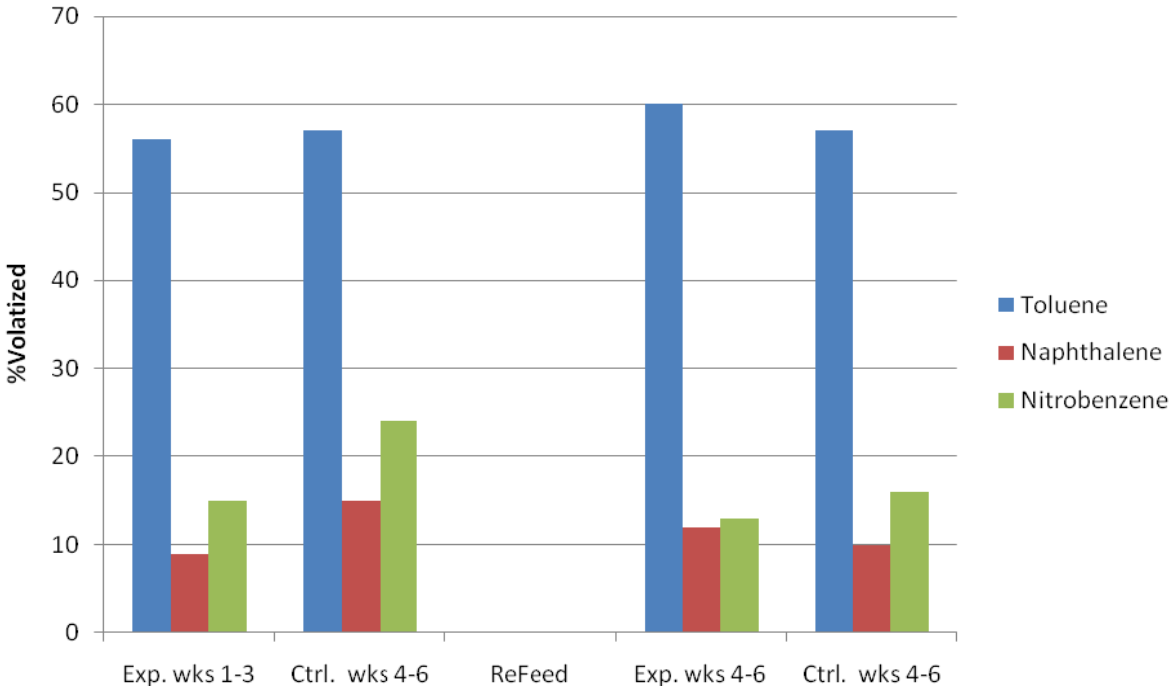

Figure 9. Percent loss of 500ppm each of NT and 50ppm NoBz, lost to volatilization in box reactors supplemented with atrazine, as determined by VOC trap extractions.

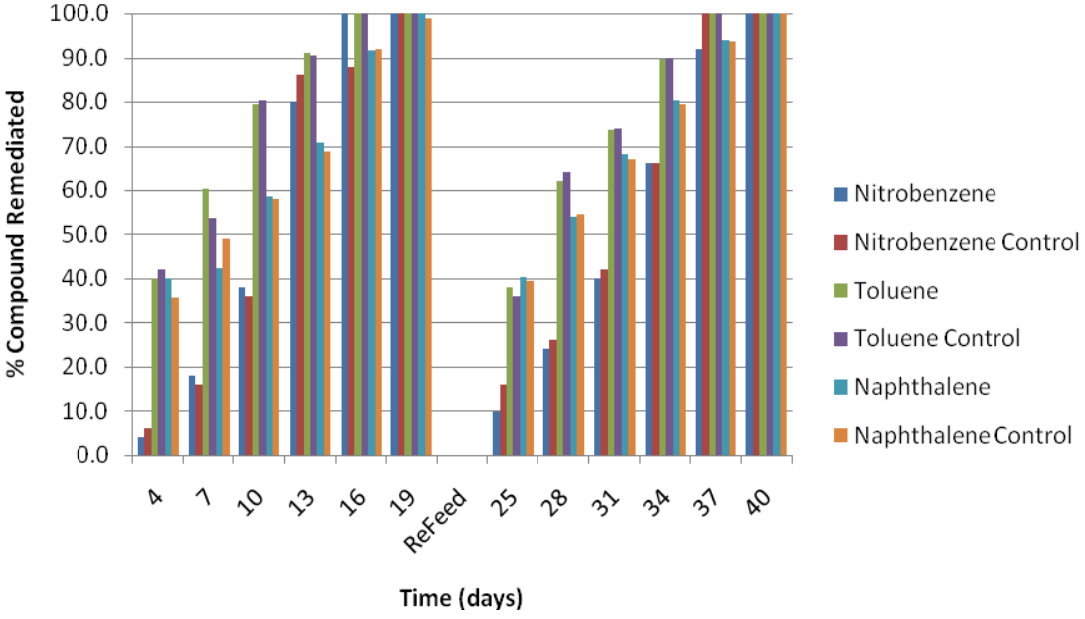

Figure 10. Percent remediated of 500ppm each of NT and 50ppm of NoBz, supplemented with atrazine in box reactors.

Degradation of 1000ppm each of NT and 100ppm of NoBz in Box Reactors Supplemented with either Cyanuric Acid, Melamine, or Atrazine

Figure 11 shows the degradation of 1000ppm each of NT, and 100ppm of NoBz within box reactors supplemented with cyanuric acid over a four week period. The absolute value of the slope in table 8 shows that the rate of nitrobenzene and naphthalene degradation in the experimental box before and after the reefed was faster than the control counterpart. However, the rate of toluene degradation in the experimental box before and after the reefed was slower than the control box. Volatilization of NT before and after the reefed in experimental and control boxes is similar. Likewise, volatilization of nitrobenzene in both experimental and control boxes after the reefed is similar, but volatilization of nitrobenzene in the experimental box was about 10% greater than the control box before the reefed (figure 12). Figure 13 shows that toluene in the experimental and control boxes, and nitrobenzene in the experimental box were completely remediated after the initial feed, while naphthalene in the experimental and control boxes and nitrobenzene in the control box were not completely removed from the environment. After the reefed the naphthalene in the control box had not been completely remediated.

Figure 14 shows the degradation of 1000ppm each of NT and 100ppm of NoBz supplemented with melamine observed over a four week period. The rate of compound degradation within the boxes, as determined by the slope in table 9, indicates that nitrobenzene in the control box was degraded faster than the nitrobenzene in the experimental box before the reefed, while toluene and naphthalene in the experimental box had a faster rate of degradation than the control box before the refeed. After the reefed, nitrobenzene and naphthalene in the experimental box was degraded faster than nitrobenzene and naphthalene in control box, while toluene in the control box was degraded slightly faster than toluene in the experimental box. Figure 15 shows that toluene is the most volatile with greater than 30% of the VOC being

remove by volatilization, followed by naphthalene and nitrobenzene which each show less than 10% being lost to volatilization. Figure 16 shows that only naphthalene in both the experimental and control boxes remained in the environments after the initial reefed. At the end of the degradation run only naphthalene in the control box remained in the environment.

Figure 17 shows the degradation of 1000ppm each of NT and 100ppm of NoBz supplemented with atrazine over four week period. NNT degradation rate, quantified by the slope of line in table 10, shows that nitrobenzene in the experimental box was degraded faster than nitrobenzene in the control box before and after the refeed. Toluene and naphthalene in the experimental box degraded faster than control the box after the initial feed, but both toluene and naphthalene in the experimental box were degraded slower than the control counterpart after the reefed. Volatilization of toluene is significantly higher in the control box before and after the reefed when using atrazine (figure 18). Figure 19 shows that when using atrazine as a nitrogen source, after the initial feed, none of the compounds have been completely removed from the environment and that after the reefed only naphthalene is left in the environment.

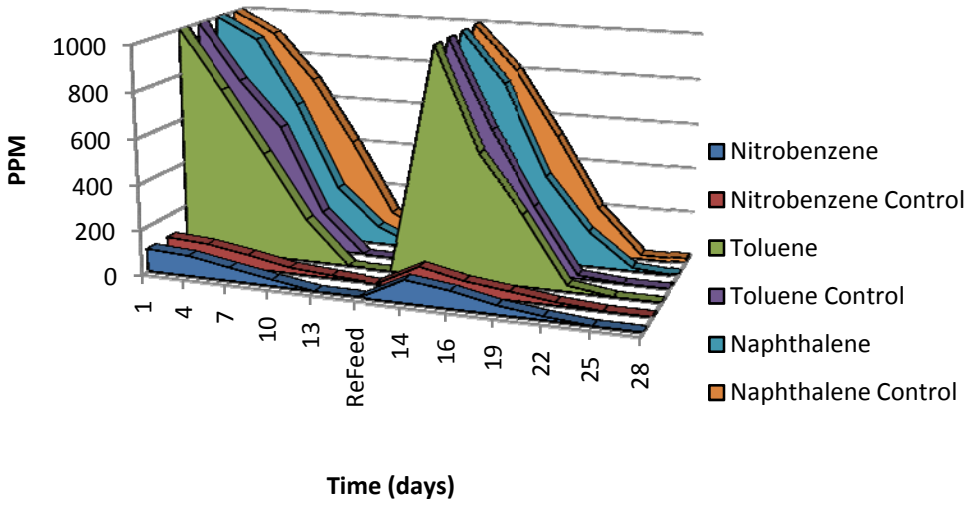

Figure 11. Degradation of 1000ppm each of NT and 100ppm of NoBz, supplemented with cyanuric acid, over a 4 week period in box reactors.

Table 8. Degradation rate of 1000 ppm each of NT and 100ppm of NoBz, supplemented with cyanuric acid, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Week 1-3	26.20	24	250	251.50	250.70	229.70
Week 4-6	21.50	20.10	316.20	327	218.20	216.90

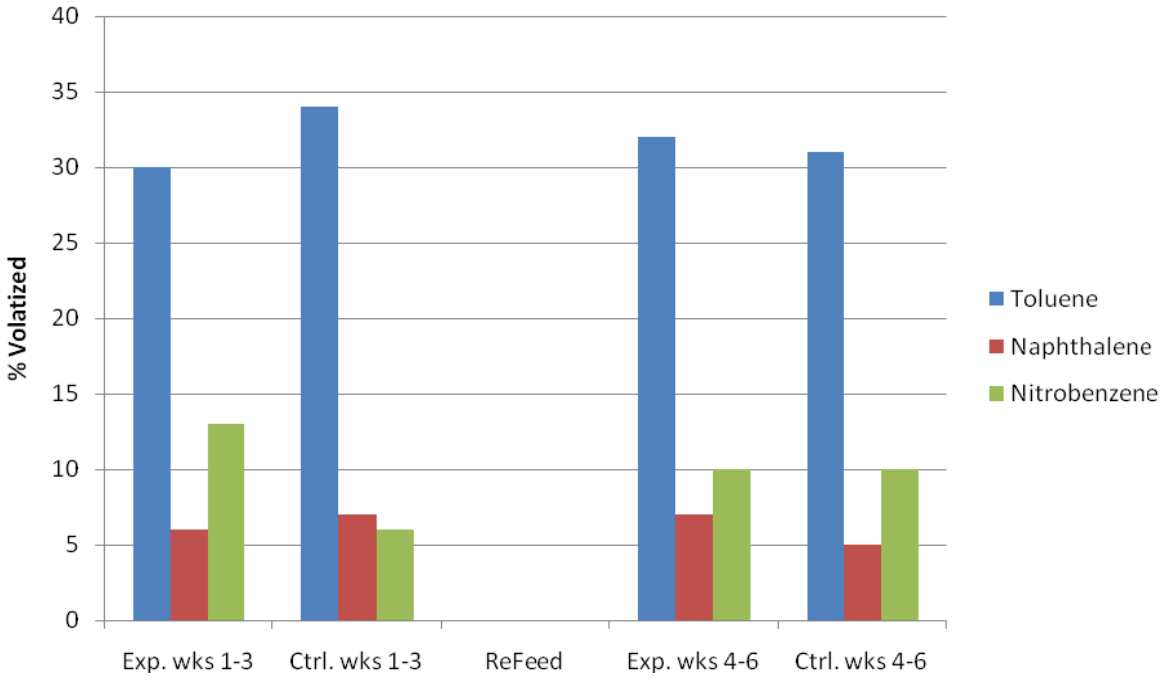

Figure 12. Percent loss of 1000ppm each of NT and 100ppm NoBz, lost to volatilization in box reactors supplemented with cyanuric acid, as determined by VOC trap extractions.

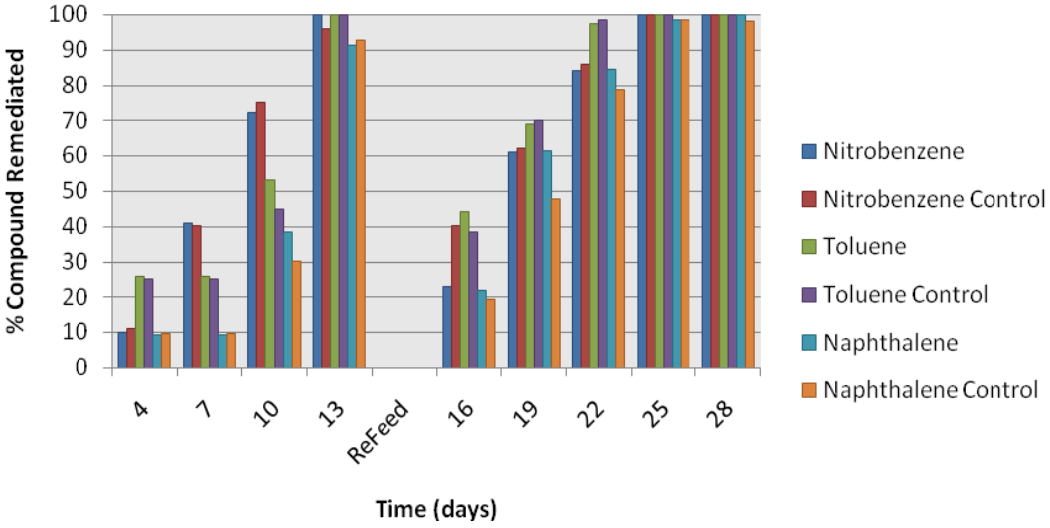

Figure 13. Percent remediated of 1000ppm each of NT and 100ppm of NoBz, supplemented with cyanuric acid in box reactors.

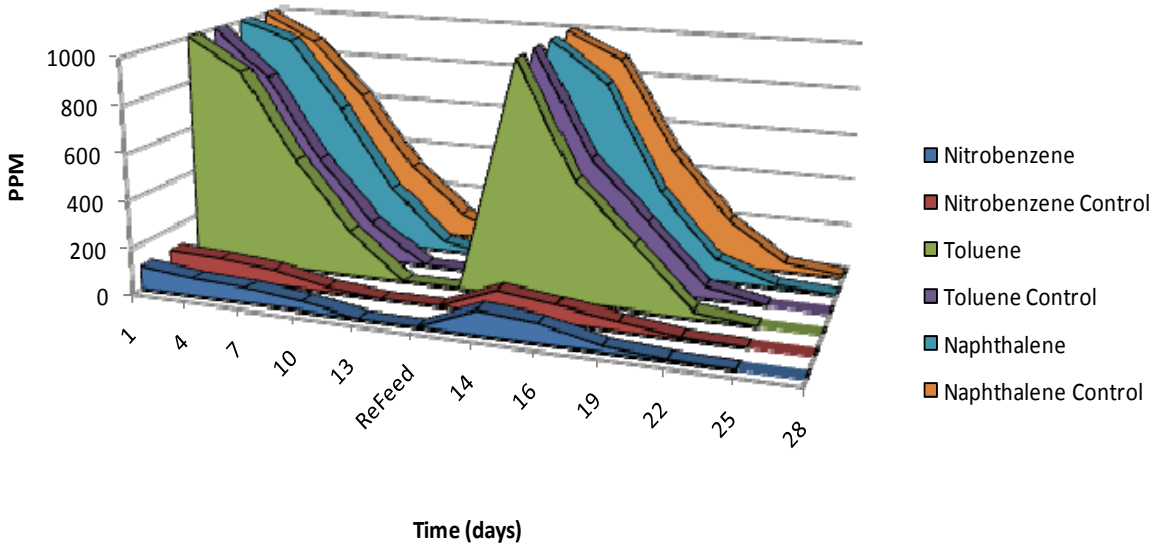

Figure 14. Degradation of 1000ppm each of NT and 100ppm of NoBz, supplemented with melamine, over a 4 week period in box reactors.

Table 9. Degradation rate of 1000 ppm each of NT and 100ppm of NoBz, supplemented with melamine, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Week 1-3	23.57	25.37	265.60	261.60	258	235.70
Week 4-6	27.50	26.20	306.80	308.40	269.48	242

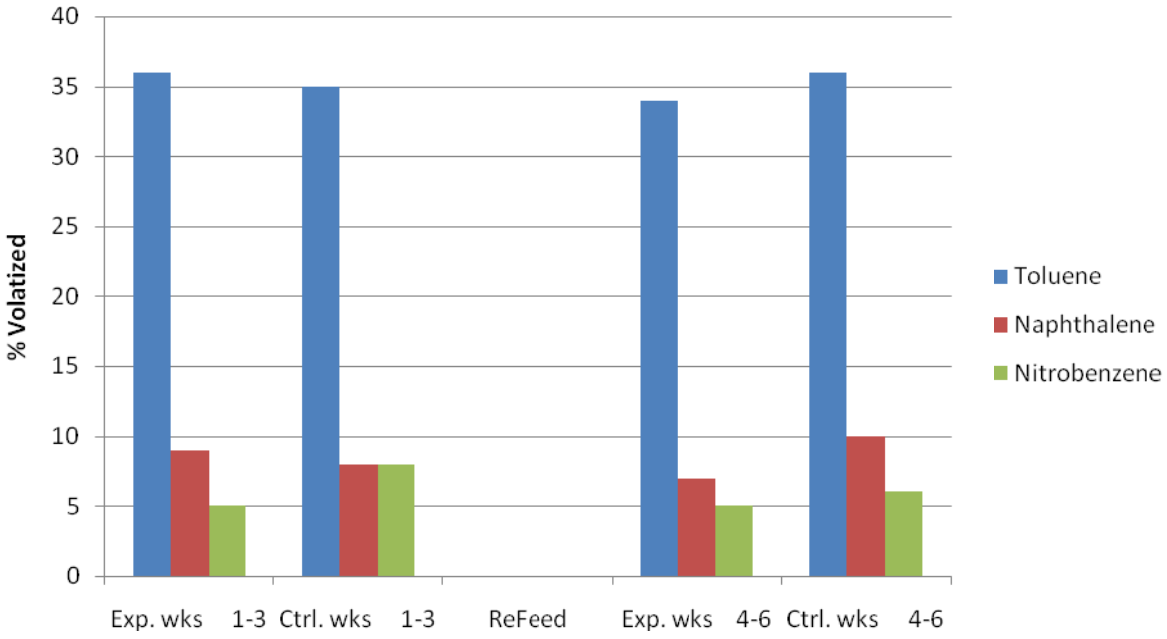

Figure 15. Percent loss of 1000ppm each of NT and 100ppm of NoBz, lost to volatilization in box reactors containing melamine, as determined by VOC trap extractions.

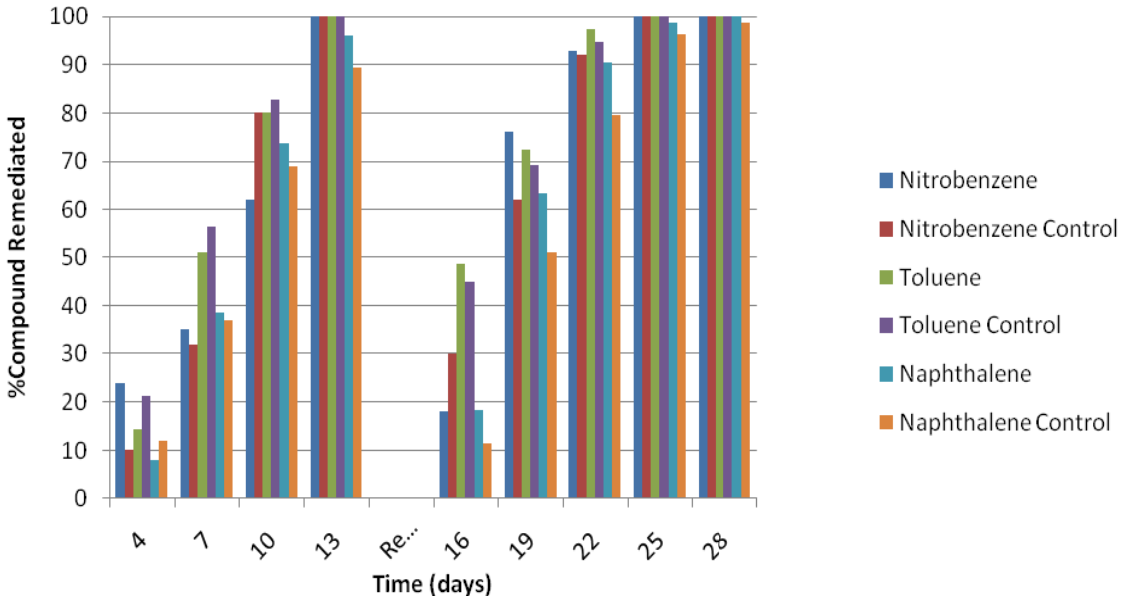

Figure 16. Percent remediated of 1000ppm each of NT and 100ppm of NoBz, supplemented with melamine in box reactors.

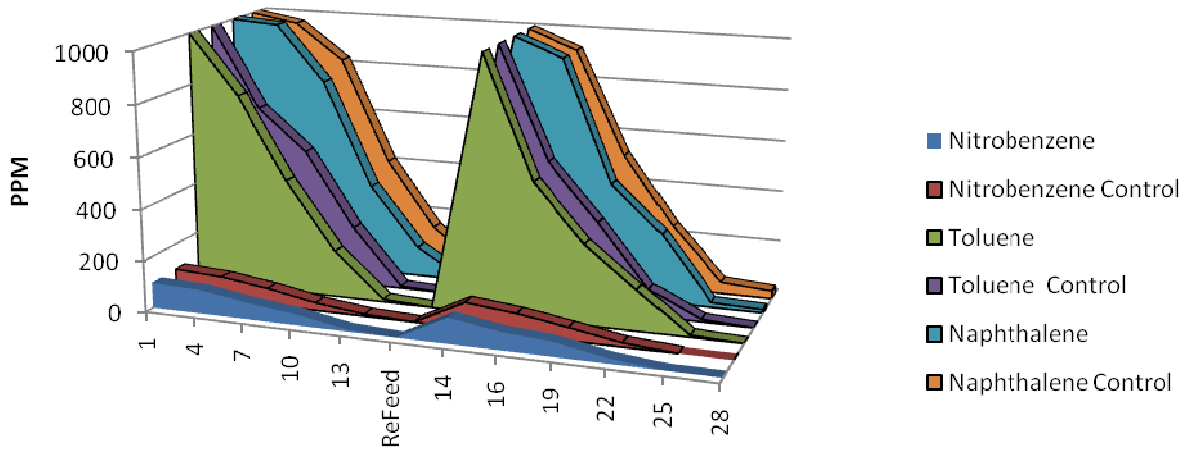

Figure 17. Degradation of 1000ppm each of NT and 100ppm of NoBz, supplemented with atrazine, over a 4 week period in box reactors.

Table 10. Degradation rate of 1000 ppm each of NT and 100ppm of NoBz, supplemented with atrazine, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Week 1-3	24.31	23	256.50	243.90	240.20	224.80
Week 4-6	26.40	25	221.40	223.88	222.80	241.71

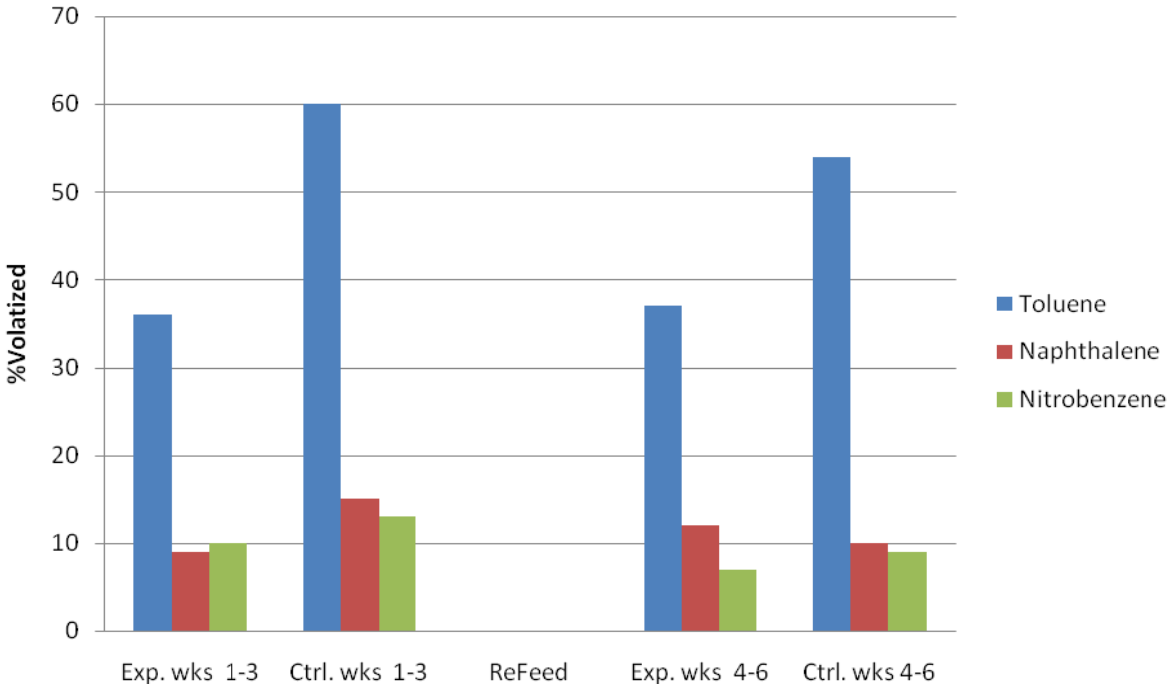

Figure 18. Percent loss of 1000ppm each of NT and 100ppm of NoBz, lost to volatilization in box reactors supplemented with atrazine, as determined by VOC trap extractions.

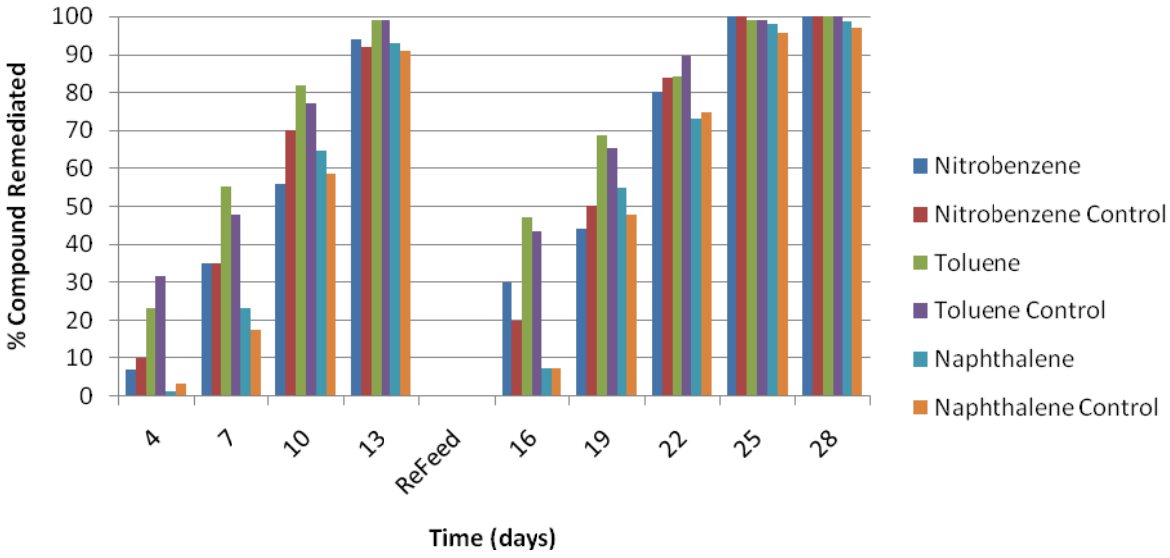

Figure 19. Percent remediated of 1000ppm each of NT and 100ppm of NoBz, supplemented with atrazine in box reactors.

Degradation of 2000ppm each of NT and 200ppm of NoBz in Box Reactors Supplemented with either Cyanuric acid, Melamine, or Atrazine

Figure 20 shows the degradation of 2000ppm each of NT, and 200ppm of NoBz in experimental and control box reactors supplemented with cyanuric acid over a four week period. The absolute value of the slope of the line shows that the degradation rate NNT was faster after the reefed as compared to the initial feed (table 11). Nitrobenzene and naphthalene in the control box were degraded faster than nitrobenzene and naphthalene in the experimental box before and after the refeed. Toluene in the experimental box was degraded faster than in the control box before and after the refeed. Figure 21 shows that NNT volatized slightly more before the refeed as opposed to after the refeed. Figure 22 shows that nitrobenzene in the experimental box and naphthalene in the experimental and control boxes were still present within the environment, after the initial feed. However, after the reefed naphthalene was the only compound still present after the degradation run.

The degradation of 2000ppm each of NT and 200 ppm of NoBz supplemented with melamine, over a four week period, is shown in figure 23. Results from the slope of the line, in table 12, show that nitrobenzene in the control box was being degraded faster than nitrobenzene in the experimental box before and after the reefed whereas toluene present in the experimental box was being degraded faster than toluene in the control box before and after the refeed. Naphthalene in the control box was degraded at a faster rate than naphthalene in the experimental box after the initial feed, and degraded slower after the reefed. Volatilization results of NNT seen in reactors containing melamine show that more nitrobenzene volatized in the control box before the refeed as opposed to after the refeed (figure 24). NT was volatized about the same before and after the refeed in both experimental and control boxes. Figure 25

shows that naphthalene was the only compound present within the environment at the end of the degradation runs.

Degradation of 2000ppm each of NT and 200ppm of Nobz supplemented with of atrazine is shown in figure 26. The rate of degradation of nitrobenzene and naphthalene in the experimental box and control box before the refeed was about the same (table 13). After the reefed, nitrobenzene and naphthalene in the experimental box was removed from the environment faster than their control counterpart. Toluene present within the experimental box before the refeed was degraded faster than toluene in control box. After the refeed, toluene in control box showed the same rate of degradation as in experimental box. Volatilization results show that on average, inoculated boxes had higher volatility rates of naphthalene and nitrobenzene in the experimental box as compared to the control box (figure 27). More nitrobenzene and naphthalene persisted in the environment before the refeed as opposed to after the reefed where lower amount were present (figure 28). Toluene was the only compound completely removed after the initial feed and reefed. Nitrobenzene and naphthalene were still present within both experimental and control boxes once the degradation runs were complete.

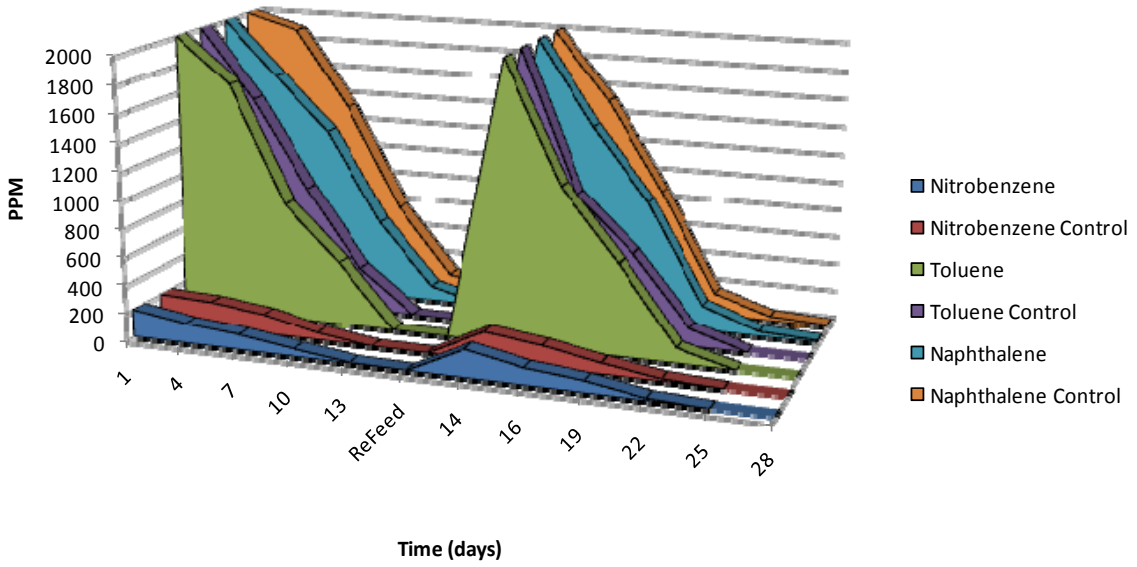

Figure 20. Degradation of 2000ppm each of NT and 200ppm of NoBz, supplemented with cyanuric acid, over a 4 week period in box reactors.

Table 11. Degradation rate of 2000 ppm each of NT and 200ppm of NoBz, supplemented with cyanuric acid, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental	Nitrobenzene Control Box	Toluene Experimental	Toluene Control Box	Naphthalene Experimental	Naphthalene Control Box
Week 1-3	40.72	45.35	522.50	519.20	478.21	483
Week 4-6	50.40	53.30	616.50	599.90	515.50	529.70

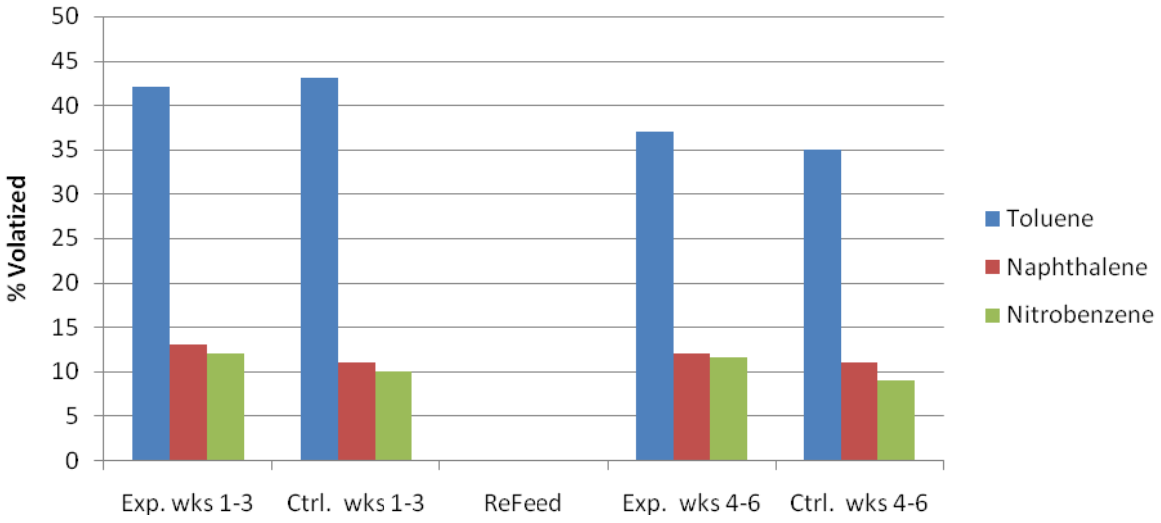

Figure 21. Percent loss of 2000ppm each of NT and 200ppm NoBz, lost to volatilization in box reactors supplemented with cyanuric acid, as determined by VOC trap extractions.

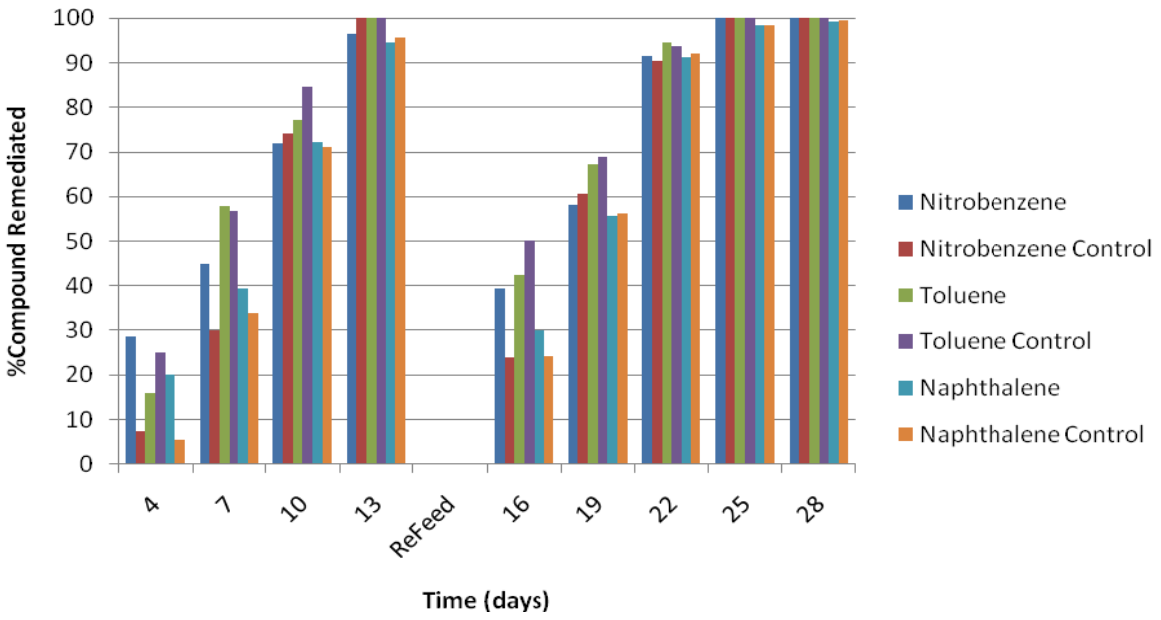

Figure 22. Percent remediated of 2000ppm each of NT and 200ppm of NoBz, supplemented with cyanuric acid in box reactors.

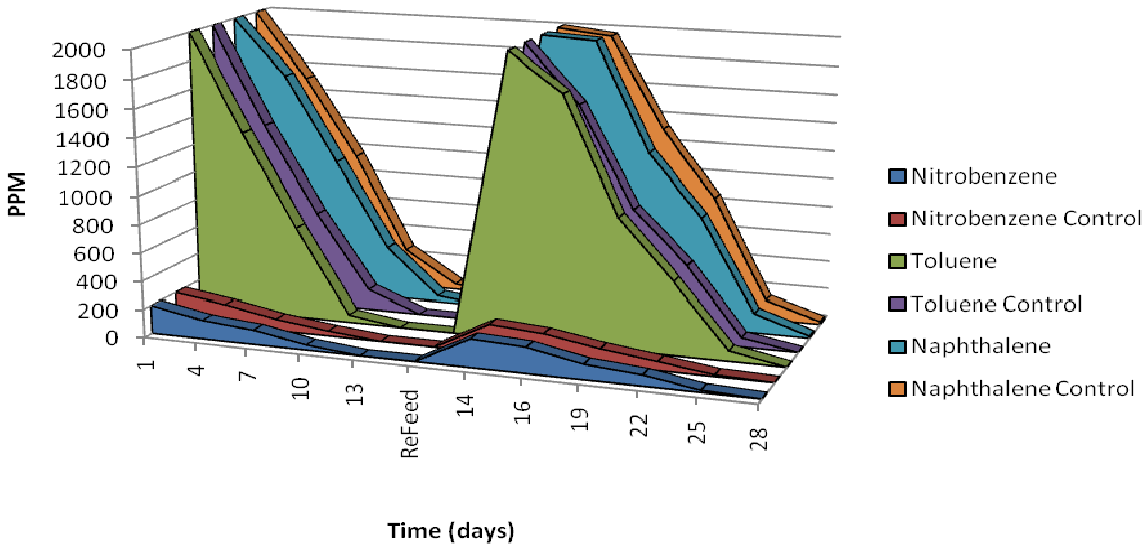

Figure 23. Degradation of 2000ppm each of NT and 200ppm of NoBz, supplemented with melamine, over a 4 week period in box reactors.

Table 12. Degradation rate of 2000 ppm each of NT and 200ppm of NoBz, supplemented with melamine, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Week 1-3	49.60	51.40	525.80	515.30	513.90	517
Week 4-6	43.90	44.90	507.10	493.20	453.94	452.37

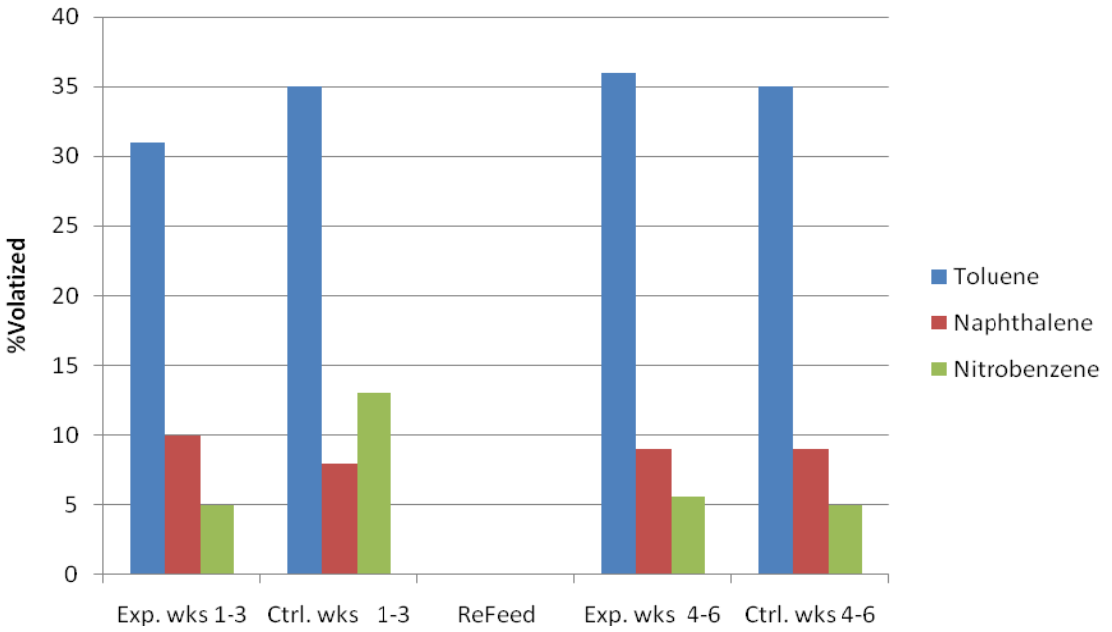

Figure 24. Percent loss of 2000ppm each of NT and 200ppm of NoBz, lost to volatilization in box reactors supplemented with melamine, as determined by VOC traps.

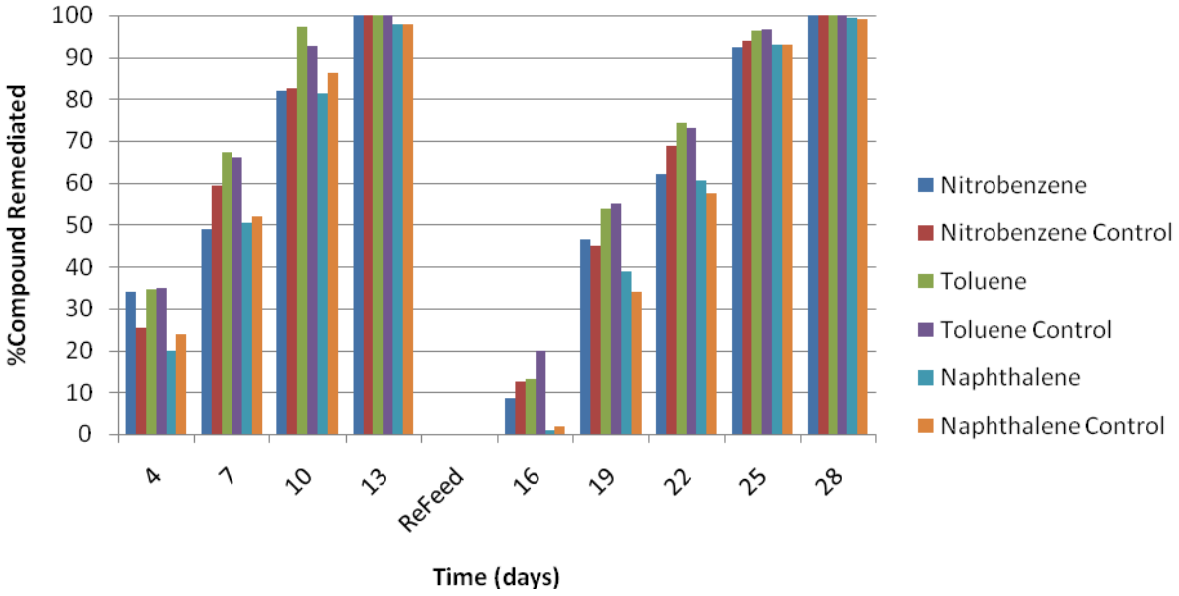

Figure 25. Percent remediated of 2000ppm each of NT and 200ppm of NoBz, supplemented with melamine in box reactors.

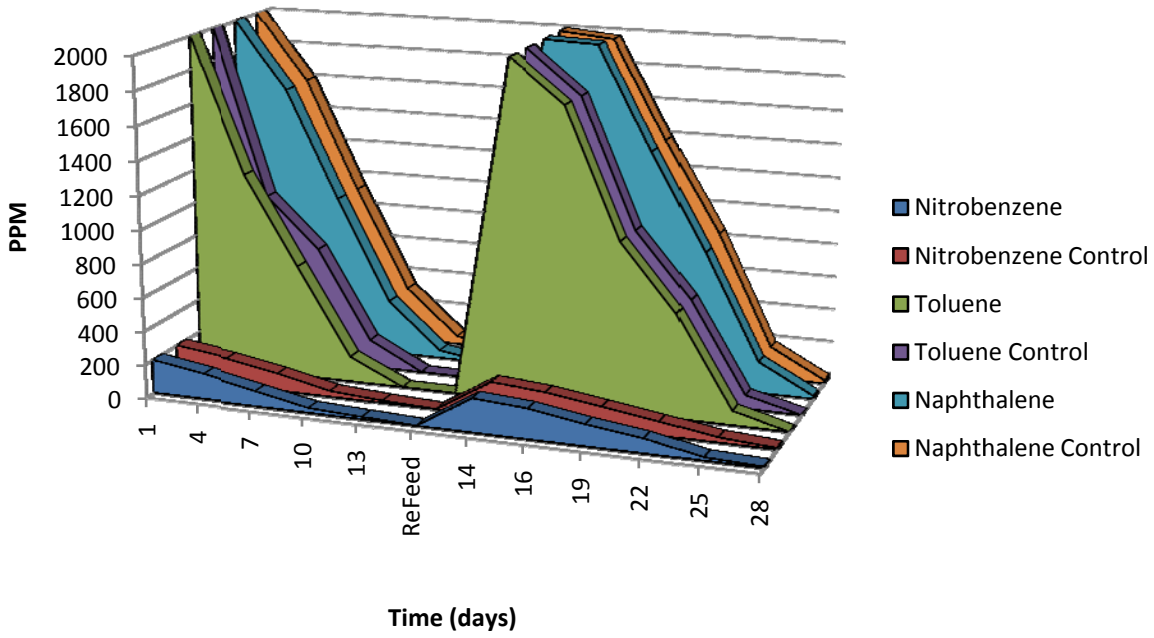

Figure 26. Degradation of 2000ppm each of NT and 200ppm of NoBz, supplemented with atrazine, over a 4 week period in box reactors.

Table 13. Degradation rate of 2000ppm each of NT and 100ppm of NoBz, supplemented with atrazine, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Week 1-3	49.4	49.8	607	580.01	517	517
Week 4-6	49.37	48.17	446.71	446.83	453.85	451.14

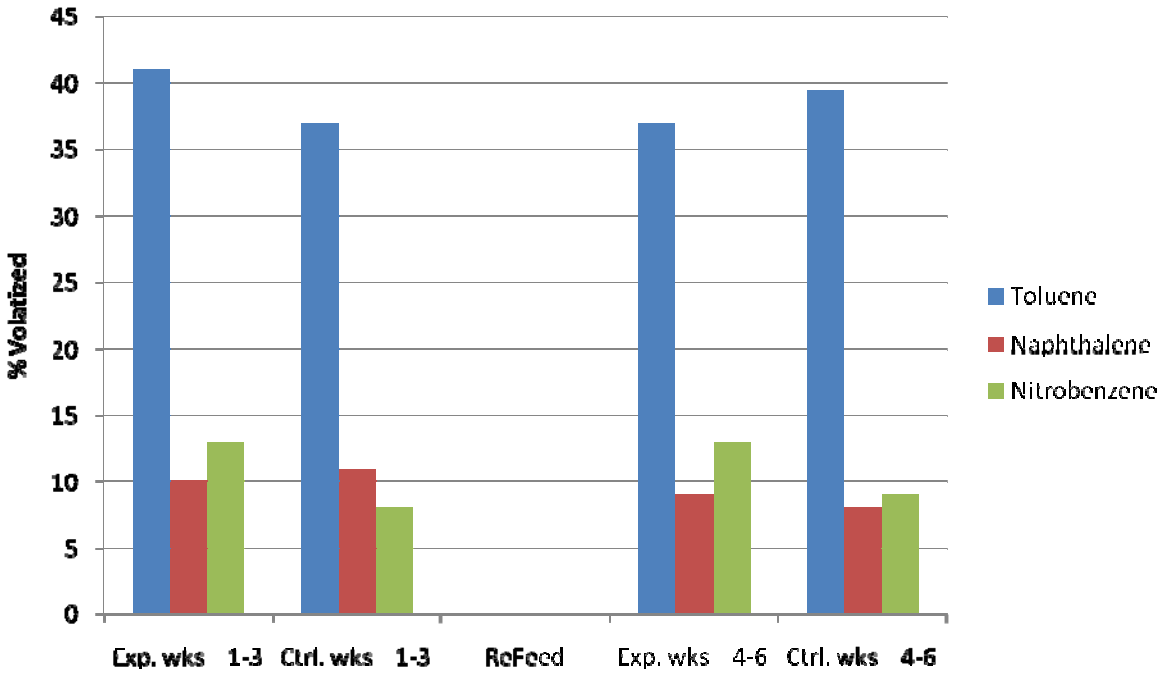

Figure 27. Percent loss of 2000ppm each of NT and 200ppm of NoBz, lost to volatilization in box reactors containing atrazine, as determined by VOC trap extractions.

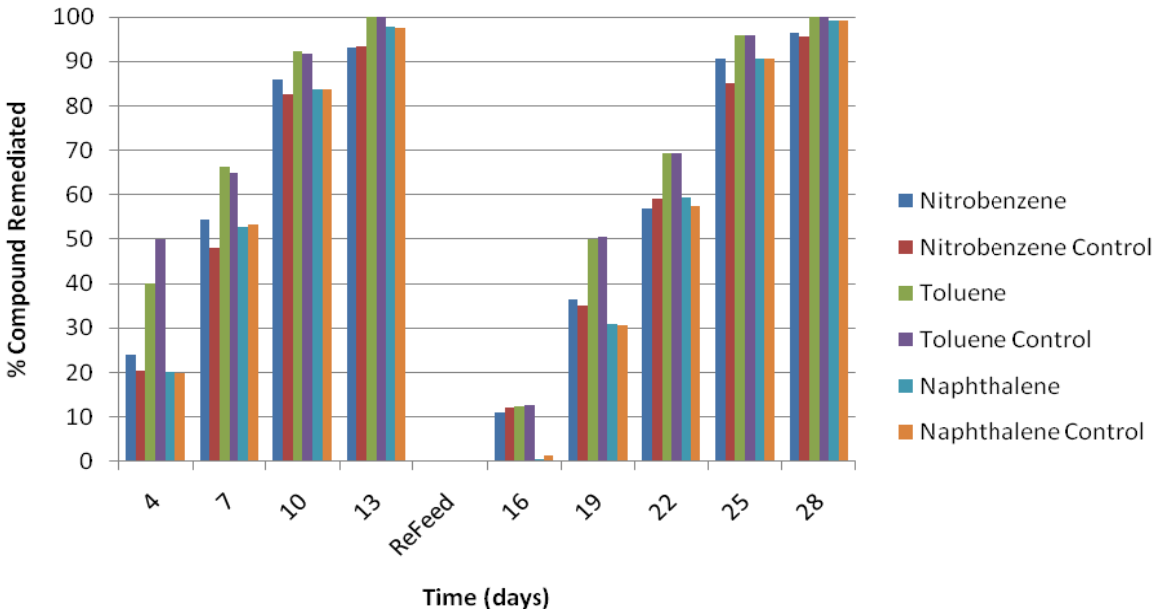

Figure 28. Percent remediated of 2000ppm each of NT and 200ppm of NoBz, supplemented with atrazine in box reactors.

Degradation of 3000ppm each of NT and 300ppm of NoBz in Box Reactors Supplemented with either Cyanuric Acid, Melamine, or Atrazine

Figure 29 shows the degradation of 3000ppm each of NT and 300 ppm of NoBz supplemented with cyanuric acid, over a 4 week period. The slope of the line, table 14, shows that nitrobenzene and toluene in the experimental box was degraded slower than in the control box before and after there refeed. Naphthalene degradation was the same in both experimental and control boxes before the reefed and greater in the experimental box after the reefed. NNT removal, attributed to volatilization, is roughly equivalent before and after the refeed (figure 30). Figure 31 shows that toluene was the only compound completely removed after the initial feed, whereas only naphthalene was present after the reefed. Compound removal was significantly greater after the reefed than before.

Degradation of NNT at concentrations of 3000ppm each of NT and 300ppm of NoBz supplemented with melamine, over a 4 week period, are illustrated in figure 32. Table 15 shows that nitrobenzene was degraded faster in the experimental box than in the control box before the reefed. After the reefed, the degradation rate of nitrobenzene in the experimental box was slower than in the control box. Toluene and naphthalene in the control box degraded at a faster rate than in the experimental box before and after the refeed. There is no difference between volatilization of NNT before and after the refeed, as shown in figure 33. Figure 34 shows that greater than 90% of all compounds were degraded before the reefed. Naphthalene was the only compound that was present after the reefed.

The degradation of 3000ppm each of NT, and 300ppm of NoBz supplemented with atrazine over a 4 week period, is shown in figure 35. The value of the slope of the line in table 16 indicates that nitrobenzene and naphthalene in the experimental box were degraded at a faster rate than the control box before and after the refeed. Toluene in the experimental box was

degraded faster than toluene in the control box before the reefed and the opposite occurred after the reefed. Figure 36 shows that NNT removal linked to volatilization is similar to what is seen in boxes containing cyanuric acid and melamine. Figure 37 shows that only toluene was completely remediated from box reactors before and after refeed. Nitrobenzene and naphthalene were still present within all boxes at the end of both the initial feed and after.

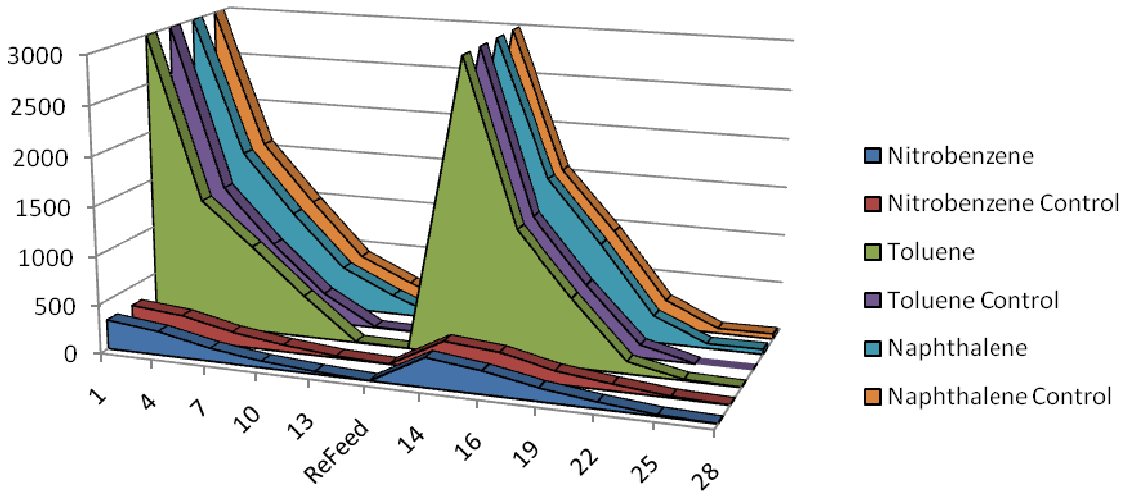

Figure 29. Degradation of 3000ppm each of NT and 300ppm of NoBz supplemented, with cyanuric acid, over a 4 week period in box reactors.

Table 14. Degradation rate of 3000ppm each of NT and 300ppm of NoBz, supplemented with cyanuric acid, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental	Toluene Control Box	Naphthalene Experimental	Naphthalene Control Box
Week 1-3	63.17	64.74	962	965.40	828	828.30
Week 4-6	73.70	75.10	853	854.60	840	838.10

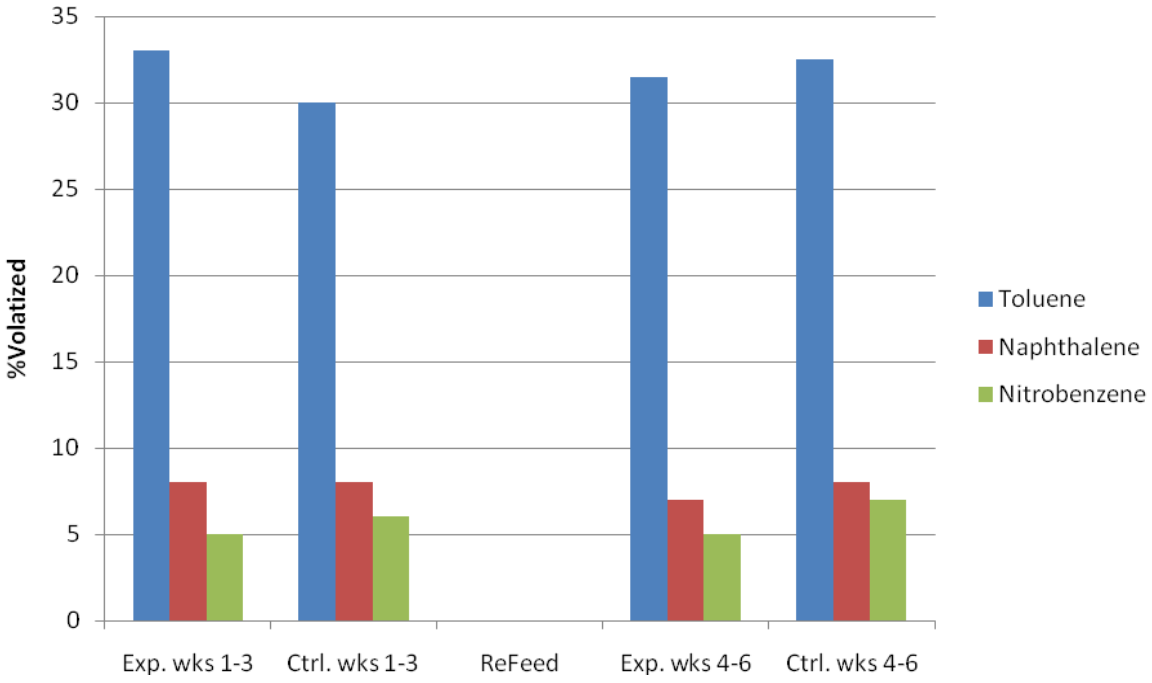

Figure 30. Percent loss of 3000ppm each of NT and 300ppm NoBz, lost to volatilization in box reactors supplemented with cyanuric acid, as determined by VOC trap extractions.

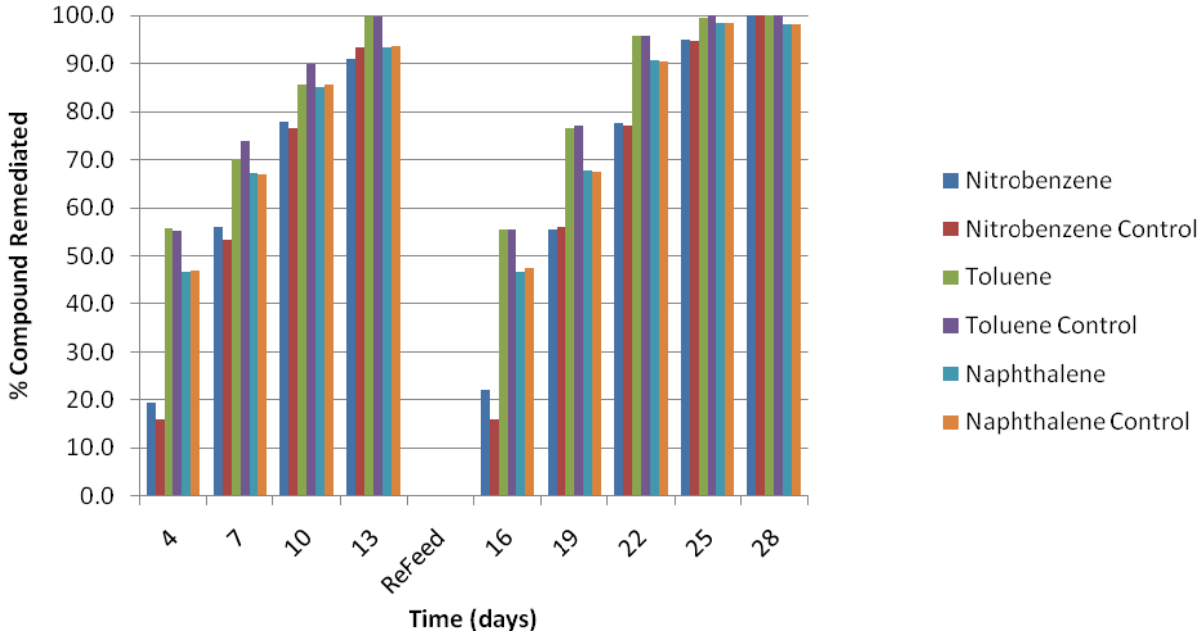

Figure 31. Percent remediated of 3000ppm each of NT and 300ppm of NoBz, supplemented with cyanuric acid in box reactors.

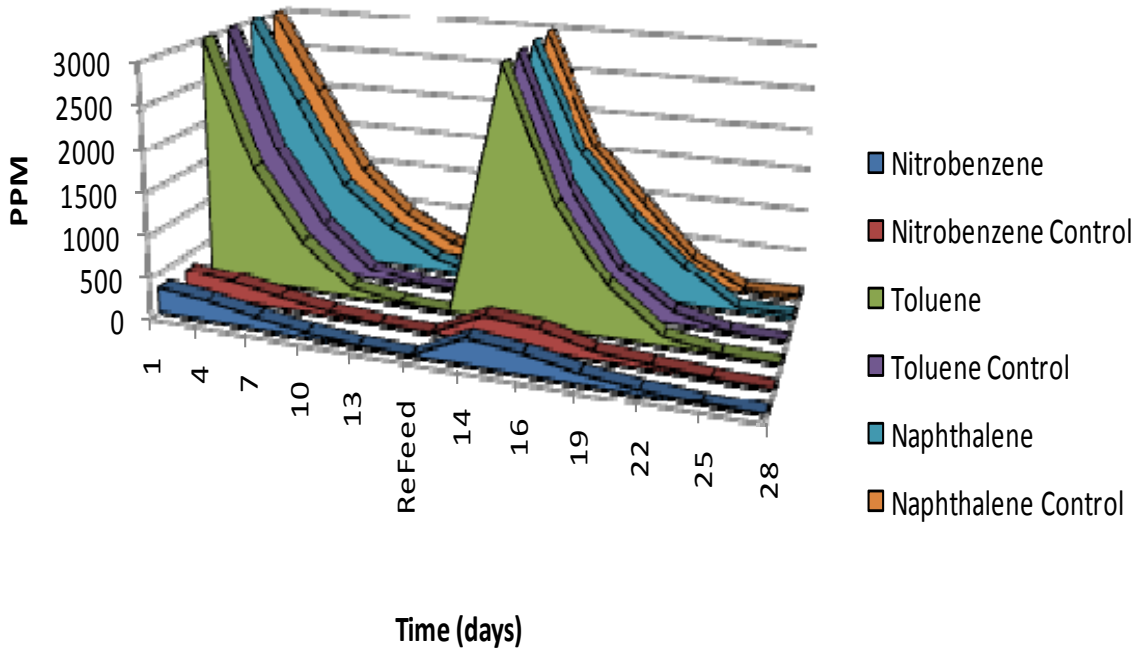

Figure 32. Degradation of 3000ppm each of NT and 300ppm of NoBz, supplemented with melamine, over a 4 week period in box reactors.

Table 15. Degradation rate of 3000ppm each of NT and 300ppm of NoBz, supplemented with melamine, as measured by the absolute value of slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Week 1-3	65.22	64.82	560.50	568.10	590.30	593.50
Week 4-6	73.80	75.30	731.50	732.90	711.90	717.60

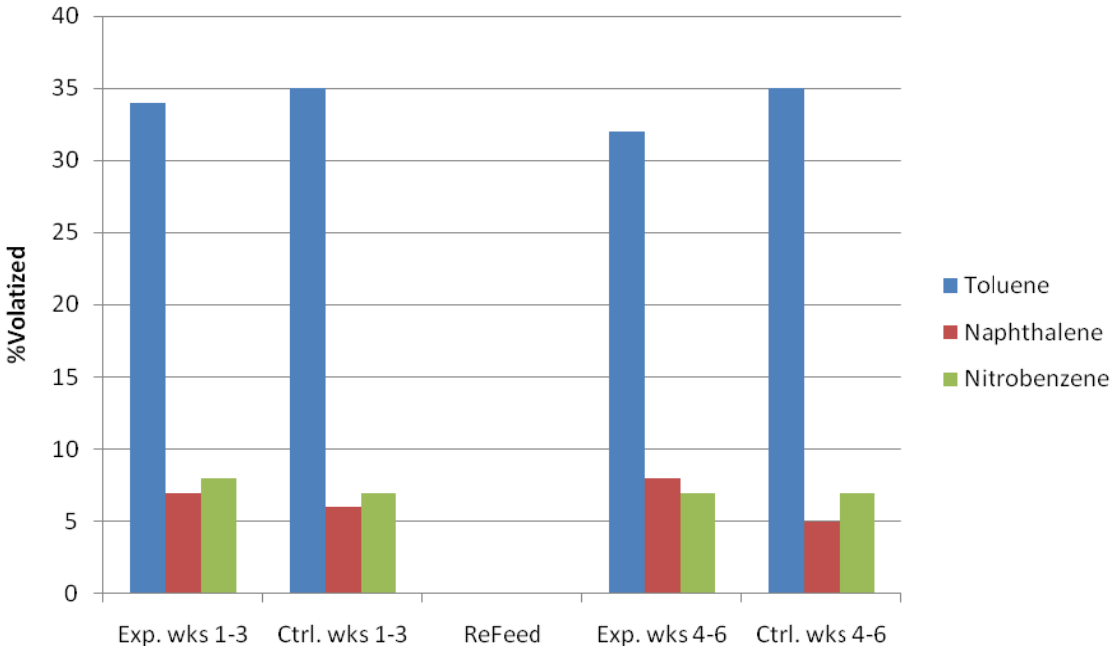

Figure 33. Percent loss of 3000ppm each of NT and 300ppm of NoBz, lost to volatilization in box reactors supplemented with melamine, as determined by VOC trap extractions.

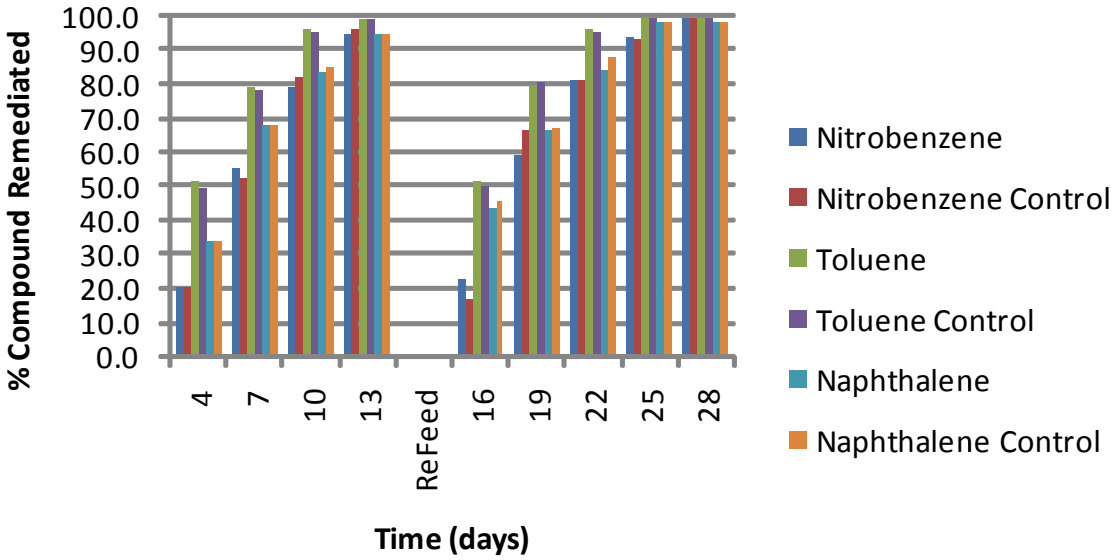

Figure 34. Percent remediated of 3000ppm each of NT and 300ppm of NoBz, supplemented with melamine in box reactors.

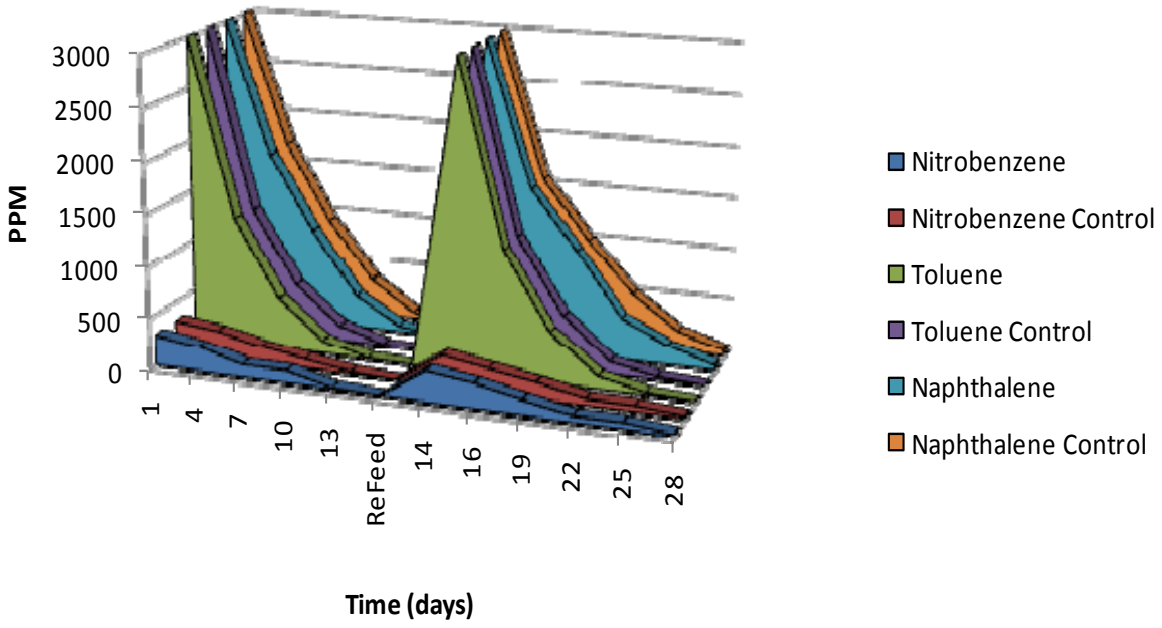

Figure 35. Degradation of 3000ppm each of NT and 300ppm of NoBz, supplemented with atrazine, over a 4 week period in box reactors.

Table 16. Degradation rate of 3000ppm each of NT and 300ppm of NoBz, supplemented with atrazine, as measured by the absolute value of the slope of the line (Δsubstrate/Δdays).

	Nitrobenzene Experimental Box	Nitrobenzene Control Box	Toluene Experimental Box	Toluene Control Box	Naphthalene Experimental Box	Naphthalene Control Box
Week 1-3	72.90	71.90	935.30	929.50	878.30	869
Week 4-6	57.14	56.77	936.10	937.30	836.80	829.60

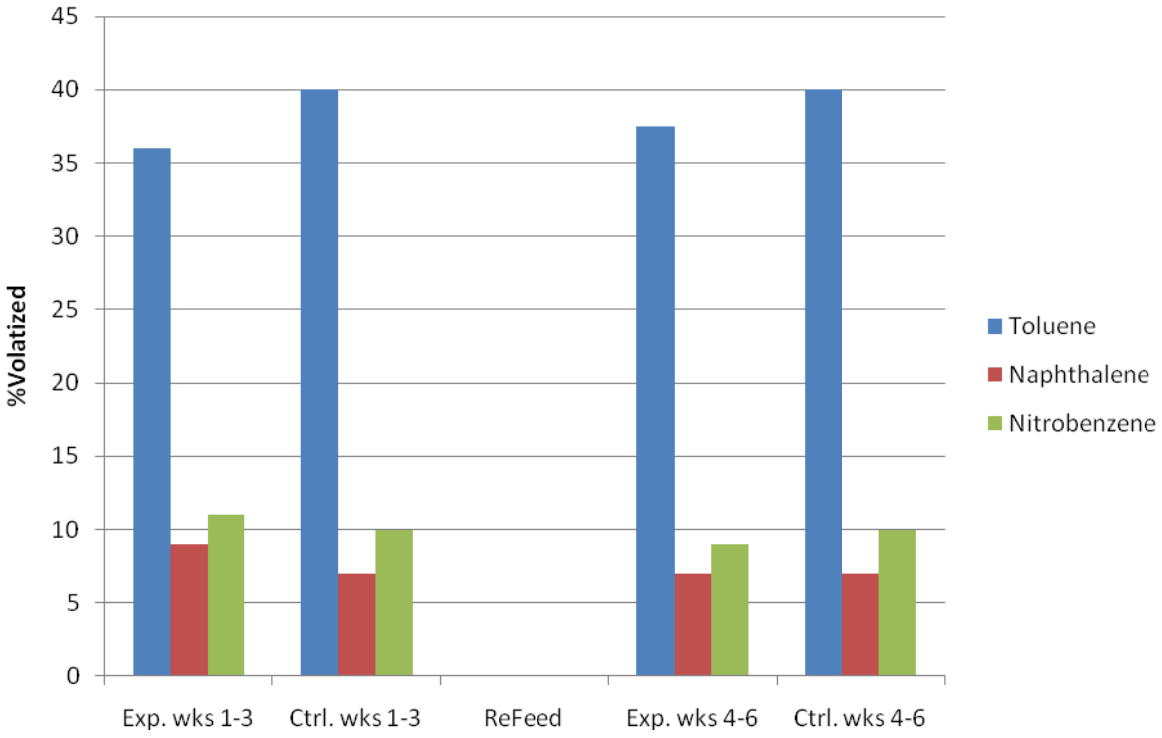

Figure 36. Percent loss of 3000ppm each of NT and 300ppm of NoBz, lost to volatilization in box reactors supplemented with atrazine, as determined by VOC trap extractions.

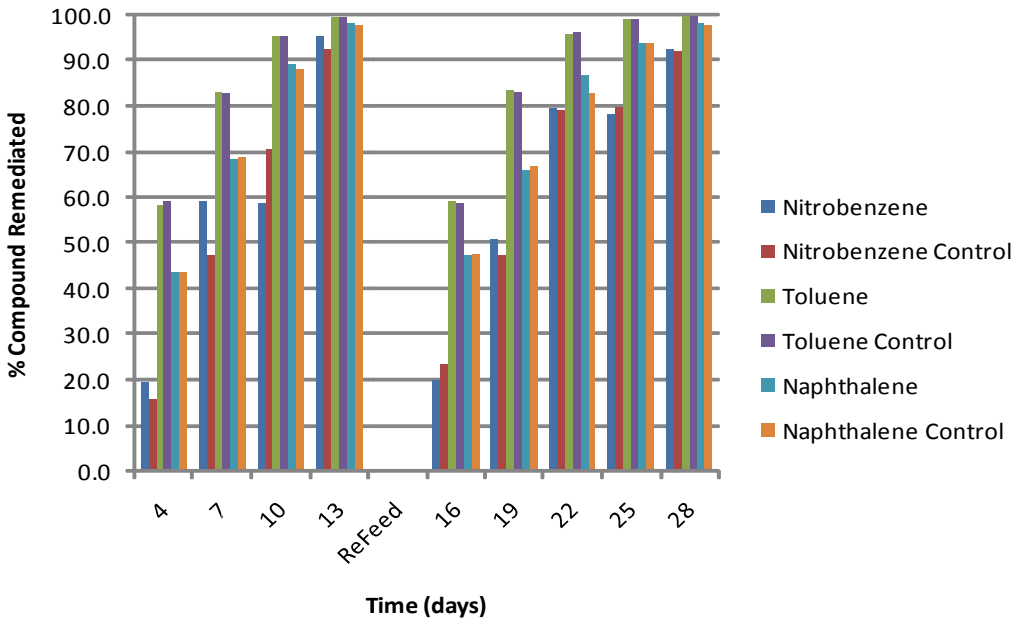

Figure 37. Percent remediated of 3000ppm each of NT and 300ppm of NoBz, supplemented with atrazine in box reactors.

Community Analysis

Community analysis studies were done within each box reactor. A combined use of 16s rDNA and 16s rRNA was utilized to study the survival and activity of inoculated organisms and the bacterial population in highly contaminated NNT reactors in the presence of cyanuric acid, melamine, and atrazine.

DAP strains 66, 119, and 662 inoculated in experimental boxes were identified by 16s rDNA analysis. The 16S rRNA gene was amplified using 338F and 907 R. The organisms were found to be most similar to *Rhodococcus amico* 42, *Aeromonas* sp. PW1 *and Pseudomonas fluorescens* (table 17). Clone libraries from bacterial 16S rRNA genes were constructed from the box reactors containing cyanuric acid, melamine, and atrazine as primary nitrogen sources in the degradation of NNT. Dominant types of genera revealed in both 16s rDNA (tables 18-23) analyses and 16S rRNA analyses (tables 24-29) were identified as key organisms involved in the process of aerobic degradation of high concentrations on NNT.

Results from 16srDNA analyses showed that in inoculated boxes SBS1, SBS2, and SBS3 only two of the three organisms from the mixed culture, *Rhodococcus amico* 42 and *Pseudomonas fluorescens,* were present in clone libraries. *Rhodococcus amico* 42 was present within clone libraries' of SBS1, SBS2, SBS3, whereas *Pseudomonas fluorescens* was only seen in the clone library of SBS2. The appearance of these strains within the clone libraries suggests that they are still present within the degrading communities. The *Aeromonas* strain inoculated into box reactors was not present in clone libraries. All six (SBS1-SBS6) box reactors showed bacterial sequences mostly related to proteobacteria, and high and low G+C gram positive bacteria. There were however some bacterial sequences within box reactors that belong to the

cytaphaga group and the extremophiles, but they were not abundant in clone libraries. Each box reactor's bacterial diversity was only partially represented by the clone libraries constructed.

16s rDNA analysis was followed by 16s rRNA analyses to show whether inoculated organisms were metabolically active within degrading communities and to observe which other genera were active during the time of degradation of high concentrations of NNT. The dominant bacterial divisions seen within 16s rDNA analysis, such as the proteobacteria and the high and low G+C gram positive bacteria were also seen in 16s rRNA analysis performed on box reactors. In SBS1R, SBS2R, and SBS3R two of the three inoculated strains are present within in all boxes. This shows that they are not only present within community, but that they are active within the degrading community.

Table 17. Morphological characteristics and identification of DAP Strains 66, 119, and 622, that were inoculated into experimental boxes.

DAP Strain #	Morphological Characteristics	Accession #	Identity	% Similarity
66	Cream, waxy colonies	AY512641	*Rhodococcus* sp. Amico42 16S ribosomal RNA gene, partial sequence	97%
119	Yellow with brown edges	DQ985285	*Aeromonas* sp. PW1 16S ribosomal RNA gene, partial sequence	98%
662	White smooth colonies	AF228366	*Pseudomonas fluorescens bv. G* 16S ribosomal RNA gene, partial sequence	98%

Table 18. Taxonomic assignment of randomly selected DNA clone sequences from the inoculated box reactor degrading NNT supplemented with cyanuric acid.

Clones	Acession #	Identity	% Similarity
SBS1-1	AY921807	Uncultured *gamma proteobacterium* clone AKYG1655 16S ribosomal RNA gene, partial sequence	99%
SBS1-2	AY913408	Uncultured forest soil bacterium clone DUNssu202 16S ribosomal RNA gene, partial sequence	97%
SBS1-3	AJ585959	*Thermococcales archaeon T30a-17* partial 16S rRNA gene, clone T30a-17	97%
SBS1-4	AF151432	Bacterium ARh1 16S ribosomal RNA gene, partial sequence	99%
SBS1-5	AY728070	Uncultured *alpha proteobacterium* clone S1-9-CL5 16S ribosomal RNA gene, partial sequence	99%
SBS1-6	DQ406734	*Rhodococcus* sp. 57 16S ribosomal RNA gene, partial sequence	99%
SBS1-7	DQ130045	Uncultured *Rhizobiales* bacterium clone LPR22 16S ribosomal RNA gene, partial sequence	96%
SBS1-8	AB272321	*Nordella oligomobilis* gene for 16S rRNA, partial sequence, strain: OT1	94%
SBS1-9	DQ140383	*Pseudomonas plecoglossicida* strain R4 16S ribosomal RNA gene, partial sequence	98%
SBS1-10	AY512641	*Rhodococcus sp. Amico42* 16S ribosomal RNA gene, partial sequence	96%
SBS1-11	AY395336	Uncultured *actinobacterium* clone EB1017 16S ribosomal RNA gene, partial sequence	96%
SBS1-12	AB212806	Gamma proteobacterium NEP68 gene for 16S rRNA, partial sequence	99%
SBS1-13	AY771747	*Pseudomonas fluorescens* clone SE12 16S ribosomal RNA gene, partial sequence	97%
SBS1-14	AB192292	*Terrimonas lutea* gene for 16S rRNA, partial sequence	95%
SBS1-15	AY512641	*Rhodococcus sp. Amico42* 16S ribosomal RNA gene, partial sequence	98%
SBS1-16	DQ406734	*Rhodococcus* sp. 57 16S ribosomal RNA gene, partial sequence	98%
SBS1-17	AY921846	Uncultured *alpha proteobacterium* clone AKYG1898 16S ribosomal RNA gene, partial sequence	98%
SBS1-18	AJ252693	*Rhizosphere soil bacterium* clone RSC-II-72, 16S rRNA gene partial	93%
SBS1-19	AB245351	*Xanthobacteraceae bacterium* Gsoil 062 gene for 16S rRNA, partial sequence	98%
SBS1-20	AY771747	*Pseudomonas fluorescens* clone SE12 16S ribosomal RNA gene, partial sequence	99%
SBS1-21	AY599707	Uncultured *Xanthomonas* sp. clone CI-9-TB8-II 16S ribosomal RNA gene, partial sequence	97%
SBS1-22	AY921872	Uncultured *delta proteobacterium* clone AKYH836 16S ribosomal RNA gene, partial sequence	98%
SBS1-23	AJ863332	Uncultured bacterium partial 16S rRNA gene, clone 4RHU22	95%
SBS1-24	AB251406	*Rhodopseudomonas* sp. TUT3631 gene for 16S rRNA, partial sequence	96%
SBS1-25	AY913242	Uncultured forest soil bacterium clone DUNssu021 16S ribosomal RNA gene, partial sequence	98%
SBS1-26	AJ224613	*Alpha proteobacterium* TA1-A1, 16S rRNA gene	99%
SBS1-27	AY512641	*Rhodococcus* sp. Amico42 16S ribosomal RNA gene, partial sequence	97%
SBS1-28	AF487431	*Rhodopseudomonas palustris* strain Wa1val1 16S ribosomal RNA gene, partial sequence	99%
SBS1-29	AB249682	*Lysobacter sp. Dae08* gene for 16S rRNA, partial sequence	92%
SBS1-30	AY913411	Uncultured forest soil bacterium clone DUNssu206 16S ribosomal RNA gene, partial sequence	95%

Table 19. Taxonomic assignment of randomly selected DNA clone sequences from the inoculated box reactor degrading NNT supplemented with melamine.

Clones	Accession #	Identity	% Similarity
SBS2-1	AB012207	*Corynebacterium sp.* gene for 16S rRNA, partial sequence	99%
SBS2-2	AJ576108	Uncultured *Serratia* sp. partial 16S rRNA gene, clone 48	98%
SBS2-3	AB246772	*Myxobacterium AT3-01* gene for 16S rRNA, partial sequence	98%
SBS2-4	AJ833647	*Byssophaga cruenta* partial 16S rRNA gene, strain DSM 14553T	98%
SBS2-5	AY494635	Uncultured *Rhodoplanes* sp. clone ALPHA6A 16S ribosomal RNA gene, partial sequence	97%
SBS2-6	AF509330	*Pseudomonas putida* strain DSM 3601 16S ribosomal RNA gene, partial sequence	99%
SBS2-7	AY728070	Uncultured *alpha proteobacterium* clone S1-9-CL5 16S ribosomal RNA gene, partial sequence	94%
SBS2-8	DQ123621	*Alpha proteobacterium CRIB-04* 16S ribosomal RNA gene, partial sequence	96%
SBS2-9	AY921872	Uncultured *delta proteobacterium* clone AKYH836 16S ribosomal RNA gene, partial sequence	91%
SBS2-10	AY921951	Uncultured *Actinobacteria* bacterium clone AKYG1106 16S ribosomal RNA gene, partial sequence	99%
SBS2-11	AY512641	*Rhodococcus sp. Amico42* 16S ribosomal RNA gene, partial sequence	96%
SBS2-12	AF005016	*Nocardioides fulvus* 16S ribosomal RNA gene, partial sequence	97%
SBS2-13	AB245351	*Xanthobacteraceae bacterium Gsoil 062* gene for 16S rRNA, partial sequence	98%
SBS2-14	DQ232614	*Leifsonia shinshuensis* 16S ribosomal RNA gene, partial sequence	94%
SBS2-15	AY772474	*Pseudomonas putida* strain NA-1 16S ribosomal RNA gene, partialsequence	96%
SBS2-16	DQ887520	*Ralstonia* sp. PNP11 16S ribosomal RNA gene, partial sequence	97%
SBS2-17	AB245340	*Myxococcales bacterium Gsoil 473* gene for 16S rRNA, partial sequence	95%
SBS2-18	AY673309	*Acidimicrobidae bacterium Ellin7143* 16S ribosomal RNA gene, partial sequence	97%
SBS2-19	AB246805	*Luteimonas sp. Q-1* gene for 16S ribosomal RNA, partial sequence	96%
SBS2-20	EF100698	*Thermomonas dokdonensis* strain *DS-58 16S* ribosomal RNA gene, partial sequence	96%
SBS2-21	AY741157	*Pseudomonas putida* strain *S18 16S ribosomal RNA gene, partialsequence*	97%
SBS2-22	X95920	*X.fragariae* 16S rRNA gene	95%
SBS2-23	DQ811841	Uncultured *gamma proteobacterium* clone MSB-4C11 16S ribosomal RNA gene, partial sequence	96%
SBS2-24	AF228366	*Pseudomonas fluorescens bv. G* 16S ribosomal RNA gene, partial sequence	97%
SBS2-25	AF361187	*Flexibacter sp. CF 1* 16S ribosomal RNA gene, partial sequence	99%
SBS2-26	AF507702	Uncultured soil bacterium clone C0133 16S ribosomal RNA gene, partial sequence	100%
SBS2-27	AF228366	*Pseudomonas fluorescens bv. G* 16S ribosomal RNA gene, partial sequence	99%
SBS2-28	AF005016	*Nocardioides fulvus* 16S ribosomal RNA gene, partial sequence	98%
SBS2-29	AY921951	Uncultured *Actinobacteria* bacterium clone AKYG1106 16S ribosomal RNA gene, partial sequence	95%
SBS2-30	AB099660	*Oligotropha carboxidovorans* gene for 16S rRNA, complete sequence, strain: S28	99%

Table 20. Taxonomic assignment of randomly selected DNA clone sequences from the inoculated box reactor degrading NNT supplemented with atrazine.

Clones	Accession #	Identity	% Similarity
SBS3-1	AB270613	*Serratia marcescens* gene for 16S rRNA, partial sequene, strain:AS-1	98%
SBS3-2	AY512641	*Rhodococcus* sp. Amico42 16S ribosomal RNA gene, partial sequence	97%
SBS3-3	AY331370	*Pseudomonas* sp. MFY152 16S ribosomal RNA gene, partial sequence	99%
SBS3-4	AB032510	*Cellulophaga lytica* gene for 16S rRNA, partial sequence,strain:IFO15986	99%
SBS3-5	AY690719	*Arthrobacter* sp. MK11 16S ribosomal RNA gene, partial sequence	98%
SBS3-6	D49423	*Pseudomonas riboflavina* gene for 16S ribosomal RNA, partial sequence	97%
SBS3-7	AB078066	*Flexibacter sancti* gene for 16S rRNA, strain:IFO 15057	94%
SBS3-8	AB288061	*Rhodococcus boritolerans* gene for 16S ribosomal RNA, partial sequence, strain: BTM-1c	99%
SBS3-9	DQ333356	*Raoultella planticola* strain 33-4ch 16S ribosomal RNA gene, partial sequence	100%
SBS3-10	AY921951	Uncultured *Actinobacteria* bacterium clone AKYG1106 16S ribosomal RNA gene, partial sequence	98%
SBS3-11	AM237392	*Micrococcus luteus* partial 16S rRNA gene, isolate OS-159	96%
SBS3-12	AB096215	*Gamma proteobacterium Y-134* gene for 16S rRNA, partial sequence	98%
SBS3-13	DQ991163	*Serratia grimesii* 16S ribosomal RNA gene, partial sequence	97%
SBS3-14	AJ784812	*Pseudomonas aeruginosa* partial 16S rRNA gene, strain HXN-400	95%
SBS3-15	AY512641	*Rhodococcus* sp. Amico42 16S ribosomal RNA gene, partial sequence	98%
SBS3-16	AB019582	*Lysobacter antibioticus* gene for 16S rRNA, partial sequence	99%
SBS3-17	AJ833647	*Byssophaga cruenta* partial 16S rRNA gene, strain DSM 14553T	98%
SBS3-18	AF494540	*Burkholderia sp. BPC2 16S ribosomal RNA gene, partial sequence*	98%
SBS3-19	AJ002091	*Streptomyces thermotolerans* 16S rRNA gene, partial	100%
SBS3-20	DQ140383	*Pseudomonas plecoglossicida* strain R4 16S ribosomal RNA gene,partial sequence	98%
SBS3-21	AJ244722	*Xanthomonas-like* sp. V4.BO.41 partial 16S rRNA gene	98%
SBS3-22	AJ519989	*Thermomonas brevis* 16S rRNA gene, type strain LMG 21746T	94%
SBS3-23	DQ191178	*Lysobacter daejeonensis* 16S ribosomal RNA gene, partial sequence	99%
SBS3-24	AJ833647	*Myxobacterium* AT3-01 gene for 16S rRNA, partial sequence	98%
SBS3-25	AY136537	*Pseudomonas* sp. V-O7-3 16S ribosomal RNA gene, partial sequence	98%
SBS3-26	AF055333	*Mycobacterium farcinogenes* 16S ribosomal RNA gene, partial sequence	99%
SBS3-27	DQ831003	*Klebsiella* sp. LB-2 16S ribosomal RNA gene, partial sequence	99%
SBS3-28	AY580068	*Burkholderia* sp. 418 16S ribosomal RNA gene, partial sequence	98%
SBS3-29	AB247644	*Micrococcus thailandicus* gene for 16S rRNA, partial sequence	94%
SBS3-30	DQ490435	*Nocardiaceae bacterium* KVD-1790-12 16S ribosomal RNA gene, partial sequence	98%

Table 21. Taxonomic assignment of randomly selected DNA clone sequences from the uninoculated box reactor degrading NNT supplemented with cyanuric acid.

Clones	Accesion #	Idenitity	% Similarity
SBS4-1	AB162802	*Nocardia vinacea* gene for 16S rRNA, partial sequence, strain:IFM	94%
SBS4-2	AY331350	*Pseudomonas* sp. MFY65 16S ribosomal RNA gene, partial sequence	97%
SBS4-3	AM110933	*Bacillus subtilis* partial 16S rRNA gene, isolate B-1171	99%
SBS4-4	AB127968	*Micrococcus* sp. YGJ1 gene for 16S rRNA, partial sequence	96%
SBS4-5	AY367010	*Burkholderia* sp. Ch3-5 16S ribosomal RNA gene, partial sequence	99%
SBS4-6	AY922111	Uncultured *gamma proteobacterium* clone AKYH1436 16S ribosomal RNA gene, partial sequence	99%
SBS4-7	DQ356902	*Pseudomonas putida* strain P19 16S ribosomal RNA gene, partial sequence	99%
SBS4-8	AM110946	*Bacillus subtilis* partial 16S rRNA gene, isolate B-3065	100%
SBS4-9	AY635893	*Flavobacterium* sp. AMS2 16S ribosomal RNA gene, partial sequence	98%
SBS4-10	AY771747	*Pseudomonas* sp. KOPRI 20902 16S ribosomal RNA gene, partial sequence	100%
SBS4-11	AB188781	Uncultured *delta proteobacterium* gene for 16S rRNA, partial sequence	99%
SBS4-12	AY914072	*Corynebacterium* sp. R-4 16S ribosomal RNA gene, partial sequence	100%
SBS4-13	AM412214	*Arthrobacter* sp. Z63zhy 16S rRNA gene, isolate Z63zhy	100%
SBS4-14	AJ132994	*Pseudomonas* sp. isolate H171, 16S rRNA gene	99%
SBS4-15	AB250304	*Bacillus licheniformis* gene for 16S rRNA	99%
SBS4-16	AJ007005	*Pseudomonas* sp. 16S rRNA gene, partial, isolate LT1	100%
SBS4-17	AB270613	*Serratia marcescens* gene for 16S rRNA, partial sequene, strain:AS-1	97%
SBS4-18	AB098593	*Rhodococcus* sp. TUT1025 gene for 16S rRNA, partial sequence	99%
SBS4-19	AY771968	Uncultured *delta proteobacterium* clone SD27 16S ribosomal RNA gene, partial sequence	98%
SBS4-20	AY822615	*Rhodococcus* sp. P15 16S ribosomal RNA gene, partial sequence	99%
SBS4-21	DQ278854	Uncultured bacterium clone 5-30 16S ribosomal RNA gene, partial sequence	97%
SBS4-22	AY771747	*Pseudomonas* sp. KOPRI 20902 16S ribosomal RNA gene, partial sequence	98%
SBS4-23	DQ084462	*Pseudomonas* sp. CSS-1 16S ribosomal RNA gene, partial sequence	98%
SBS4-24	AY479983	*Ralstonia* sp. C1 16S ribosomal RNA gene, partial sequence	99%
SBS4-25	AM411940	*Nocardia* sp. P-5 partial 16S rRNA gene, strain P-5	98%
SBS4-26	AB079788	*Micrococcus luteu* s gene for 16S ribosomal RNA, partial sequence	95%
SBS4-27	DQ130045	Uncultured *Rhizobiales* bacterium clone LPR22 16S ribosomal RNA gene, partial sequence	96%
SBS4-28	AM259579	*Serratia marcescens* partial 16S ribosomal RNA gene, isolate MF42	96%
SBS4-29	AY725813	*Corynebacterium* sp. C20 16S ribosomal RNA gene, partial sequence	97%
SBS4-30	DQ239898	*Ralstonia mannitolilytica* strain SDV 16S ribosomal RNA gene, partial sequence	99%

Table 22. Taxonomic assignment of randomly selected DNA clone sequences from the uninoculated box reactor degrading NNT supplemented with melamine.

Clones	Accesion #	Idenitity	% Similarity
SBS5-1	AF494540	*Burkholderia* sp. BPC2 16S ribosomal RNA gene, partial sequence	96%
SBS5-2	AB079788	*Micrococcus luteus* gene for 16S ribosomal RNA, partial sequence	98%
SBS5-3	U93340	*Rhodococcus coprophilus* strain JCM 3200 16S ribosomal RNA gene partial sequence	99%
SBS5-4	AB016428	*Pseudomonas putida* gene for 16S rRNA, partial sequence	96%
SBS5-5	AF487431	*Rhodopseudomonas palustris* strain Wai1val1 16S ribosomal RNA gene, partial sequence	98%
SBS5-6	AM259579	*Serratia marcescens* partial 16S ribosomal RNA gene, isolate MF42	98%
SBS5-7	AY963303	Uncultured bacterium clone AH07 16S ribosomal RNA gene, partial sequence	99%
SBS5-8	AY534920	*Actinomycete* L5 16S ribosomal RNA gene, partial sequence	98%
SBS5-9	DQ028929	*Bacillus alkalogaya* 16S ribosomal RNA gene, partial sequence	99%
SBS5-10	AB053131	*Alcanivorax* sp. Tak-1 gene for 16S ribosomal RNA, partial sequence	100%
SBS5-11	AY745846	*Micrococcus* sp. JL-76 16S ribosomal RNA gene, partial sequence	97%
SBS5-12	AY518567	*Rhodococcus* sp. CDT3 16S ribosomal RNA gene, partial sequence	99%
SBS5-13	AB017354	*Arthrobacter s* p. RC100 gene for 16S rRNA, partial sequence	98%
SBS5-14	AJ420328	*Hydrogenophaga flava* 16S rRNA gene, strain DSM 619	100%
SBS5-15	AY502033	*Frankia* sp. strain FMr16 16S ribosomal RNA gene, partial sequence	99%
SBS5-16	AY494641	Uncultured *actinobacterium* clone ACTINO2B 16S ribosomal RNA gene, partial sequence	96%
SBS5-17	AJ784812	*Pseudomonas aeruginosa* partial 16S rRNA gene, strain HXN-400	96%
SBS5-18	AJ131637	*Rhodococcus erythropolis* 16S rRNA gene, strain DCL14, partial	98%
SBS5-19	AB116442	Uncultured *Acidobacteria* bacterium gene for 16S rRNA, partial sequence, clone: Y190	99%
SBS5-20	AM410614	*Pseudomonas* sp. 33zhy partial 16S rRNA gene, strain 33zhy	99%
SBS5-21	DQ084260	Uncultured *proteobacterium* clone JAB SASS clone 17 16S ribosomal RNA gene, partial sequence	99%
SBS5-22	AM117460	*Serratia* sp. EI2-02-16 partial 16S rRNA gene, isolate EI2-02-16	95%
SBS5-23	AB188781	Uncultured *delta proteobacterium* gene for 16S rRNA, partial sequence	98%
SBS5-24	AY512640	*Rhodococcus* sp. IA1XBOX 16S ribosomal RNA gene, partial sequence	97%
SBS5-25	AM259579	*Serratia marcescens* partial 16S ribosomal RNA gene, isolate MF42	99%
SBS5-26	AY741157	*Pseudomonas putida s* train S18 16S ribosomal RNA gene, partialsequence	100%
SBS5-27	DQ413155	*Thermomonas* sp. EMB 79 16S ribosomal RNA gene, partial sequence	100%
SBS5-28	AB184988	Uncultured bacterium gene for 16S rRNA, partial sequence,clone: TH-86	99%
SBS5-29	AJ298291	*Lysobacter enzymogenes* 16S rRNA gene, strain DSM 2043T	99%
SBS5-30	AB087854	*Streptomyces* sp. TR0126 gene for 16S rRNA, partial sequence	98%

Table 23. Taxonomic assignment of randomly selected DNA clone sequences from the uninoculated box reactor degrading NNT supplemented with atrazine.

Clones	Acession #	Identity	%Similarity
SBS6-1	EF178447	*Pseudomonas fluorescen* s strain 2R37 16S ribosomal RNA gene, partial sequence	99%
SBS6-2	DQ831003	*Klebsiella* sp. LB-2 16S ribosomal RNA gene, partial sequence	99%
SBS6-3	AF307869	*Pseudomonas putida* 5IIANH 16S ribosomal RNA gene, partial sequence	98%
SBS6-4	Y09639	*Sphingomonas asaccharolytica* partial 16S rRNA gene, strain IFO 15499-T	97%
SBS6-5	AY660963	*Azospirillum irakense* strain Rsb1 16S ribosomal RNA gene, partial sequence	98%
SBS6-6	AJ132994	*Pseudomonas* sp. isolate H171, 16S rRNA gene	99%
SBS6-7	AJ620346	*Rubrivivax indolicus* partial 16S rRNA gene, strain OU5	97%
SBS6-8	AB015606	*Burkholderia cepacia* KP24 gene for 16S rRNA, partial sequence	95%
SBS6-9	AF005005	*Nocardioides albus* 16S ribosomal RNA gene, partial sequence	100%
SBS6-10	EF208030	*Serratia marcescens* strain A3 16S ribosomal RNA gene, partial sequence	97%
SBS6-11	AB266612	*Cupriavidus* sp. KU-29 gene for 16S rRNA, partial sequence	99%
SBS6-12	DQ417330	*Pseudomonas fluorescens* strain 3B 16S ribosomal RNA gene, partial sequence	99%
SBS6-13	AY148082	*Nocardioides* sp. EN47 16S ribosomal RNA gene, partial sequence	96%
SBS6-14	AB245363	*Lysobacter ginsengisoli* gene for 16S rRNA, partial sequence, strain:Gsoil 357	98%
SBS6-15	DQ857898	*Alcaligenes faecalis* strain zjs02 16S ribosomal RNA gene	96%
SBS6-16	AF548761	Uncultured *Pseudomonas* sp. clone SR11d28 16S ribosomal RNA gene, partial sequence	100%
SBS6-17	AF068010	*Pseudomonas fluorescens* strain VUN 10,011 16S ribosomal RNA gene	99%
SBS6-18	AY345415	Bacterium K2-47 16S ribosomal RNA gene, partial sequence	98%
SBS6-19	D12657	*Cytophaga arvensicola* gene for 16S ribosomal RNA, partial sequence	98%
SBS6-20	AB245351	*Xanthobacteraceae* bacterium Gsoil 062 gene for 16S rRNA, partial sequence	98%
SBS6-21	AF005016	*Nocardioides fulvus* 16S ribosomal RNA gene, partial sequence	95%
SBS6-22	AB288061	*Rhodococcus boritolerans* gene for 16S ribosomal RNA, partial sequence, strain: BTM-1c	99%
SBS6-23	DQ145582	*Uncultured Rhodanobacter* sp. clone IAFIL35 16S ribosomal RNA gene, partial sequence	94%
SBS6-24	DQ854845	*Ralstonia* sp. SA-5 16S ribosomal RNA gene, partial sequence	96%
SBS6-25	AF494540	*Burkholderia* sp. BPC2 16S ribosomal RNA gene, partial sequence	96%
SBS6-26	AJ001343	*Nevskia ramosa* 16S rRNA sequence	98%
SBS6-27	AY331370	*Pseudomonas* sp. MFY152 16S ribosomal RNA gene, partial sequence	97%
SBS6-28	AY571838	Uncultured Pseudomonas sp. clone 446C 16S ribosomal RNA gene, partial sequence	95%
SBS6-29	AB099659	*Oligotropha carboxidovorans* gene for 16S rRNA, complete sequence, strain: S23	95%
SBS6-30	AY921807	Uncultured *gamma proteobacterium* clone AKYG1655 16S ribosomal RNA gene, partial sequence	97%

Table 24. Taxonomic assignment of randomly selected cDNA clone sequences from the inoculated box reactor degrading NNT supplemented with cyanuric acid.

Clones	Acession #	Identity	% Similarity
SBS1-R1	AJ011508	*Burkholderia* sp. (strain DhA-54) 16S rRNA gene, partial sequence	98%
SBS1-R2	AB174845	*Gamma proteobacterium* SA29-B gene for 16S rRNA, partial sequence	98%
SBS1-R3	AY741160	*Sphingomonas* sp. S31 16S ribosomal RNA gene, partial sequence	97%
SBS1-R4	DQ858962	*Rhodococcus ruber* strain Z56 16S ribosomal RNA gene, partial sequence	99%
SBS1-R5	AB272321	*Nordella oligomobilis* gene for 16S rRNA, partial sequence, strain: OT1	100%
SBS1-R6	AB288061	*Rhodococcus boritolerans* gene for 16S ribosomal RNA, partial sequence, strain: BTM-1c	99%
SBS1-R7	DQ512892	*Mycobacterium* sp. N12 16S ribosomal RNA gene, partial sequence	98%
SBS1-R8	AJ698724	*Nocardioides oleivorans* partial 16S rRNA gene, type strain DSM 16090T	96%
SBS1-R9	EF195102	*Ralstonia pickettii* isolate DiSf6 16S ribosomal RNA gene, partial sequence	97%
SBS1-R10	AY136537	*Pseudomonas* sp. V-O7-3 16S ribosomal RNA gene, partial sequence	98%
SBS1-R11	DQ451093	*Pseudomonas* sp. B1 16S ribosomal RNA gene, partial sequence	98%
SBS1-R12	Y18216	*Phenylobacterium immobile* 16S rRNA gene	100%
SBS1-R13	AF228366	*Pseudomonas fluorescens bv. G* 16S ribosomal RNA gene, partial sequence	97%
SBS1-R14	AY561541	*Sphingomonas* sp. 8b-1 16S ribosomal RNA gene, partial sequence	96%
SBS1-R15	AF127393	*Gluconacetobacter liquefaciens* isolate SRI1957 16S ribosomal RNA gene, partial sequence	97%
SBS1-R16	U37344	*Burkholderia* sp. isolate N3P2 16S ribosomal RNA (rrn) gene, partial sequence	99%
SBS1-R17	AB025790	*Burkholderia* sp. NF100 gene for 16S rRNA	96%
SBS1-R18	AY166908	*Pseudomonas* sp. NN018572 16S ribosomal RNA gene, partial sequence	97%
SBS1-R19	AF105389	*Pseudomonas* sp. PsK 16S ribosomal RNA gene, partial sequence	97%
SBS1-R20	AY580068	*Burkholderia* sp. 418 16S ribosomal RNA gene, partial sequence	97%
SBS1-R21	EF694073	*Pseudomonas fluorescens* strain yl 16S ribosomal RNA gene, partial sequence	97%
SBS1-R22	AY741160	*Sphingomonas* sp. S31 16S ribosomal RNA gene, partial sequence	98%
SBS1-R23	AY512641	*Rhodococcus* sp. Amico42 16S ribosomal RNA gene, partial sequence	98%
SBS1-R24	DQ103762	*Pseudomonas putida* 16S ribosomal RNA gene, partial sequence	100%
SBS1-R25	AB091837	*Pseudomonas fluorescens* gene for 16S ribosomal RNA, partial sequence	97%
SBS1-R26	AB294933	Uncultured *gamma proteobacterium* gene for 16S rRNA, partial sequence clone: pItb-vmat-16	98%
SBS1-R27	AY512641	*Rhodococcus* sp. Amico42 16S ribosomal RNA gene, partial sequence	100%
SBS1-R28	AB005655	*Gamma proteobacterium* ST1 gene for 16S ribosomal RNA	100%
SBS1-R29	EF362636	*Rhodococcus erythropolis* strain 5 16S ribosomal RNA gene, partial sequence	99%
SBS1-R30	AF055333	*Mycobacterium farcinogenes* 16S ribosomal RNA gene, partial sequence	99%

Table 25. Taxonomic assignment of randomly selected cDNA clone sequences from the inoculated box reactor degrading NNT supplemented with melamine.

Clones	Accession #	Identity	%Similarity
SBS2-R1	AF228366	*Pseudomonas fluorescens bv. G* 16S ribosomal RNA gene, partial sequence	98%
SBS2-R2	EU017403	*Rhodococcus* sp. BFXJ-1 16S ribosomal RNA gene, partial sequence	98%
SBS2-R3	EF538732	*Nocardia* sp. 171714 16S ribosomal RNA gene, partial sequence	97%
SBS2-R4	EU017403	*Rhodococcus* sp. BFXJ-1 16S ribosomal RNA gene, partial sequence	98%
SBS2-R5	DQ071561	*Bacillus licheniformis* strain MKU 2 16S ribosomal RNA gene, partial sequence	94%
SBS2-R6	AM778086	*Pseudomonas putida* partial 16S rRNA gene, strain KDM1	100%
SBS2-R7	EF362636	*Rhodococcus erythropolis* strain 5 16S ribosomal RNA gene, partial sequence	98%
SBS2-R8	DQ372732	*Mycobacterium* sp. K328W 16S ribosomal RNA gene, partial sequence	99%
SBS2-R9	DQ123718	Uncultured soil bacterium clone PAH-Bio-53 16S ribosomal RNA gene, partial sequence	99%
SBS2-R10	AB025790	*Burkholderia* sp. NF100 gene for 16S rRNA	100%
SBS2-R11	EF515832	*Pseudomonas aeruginosa* strain PT121 16S ribosomal RNA gene	100%
SBS2-R12	EF679186	Uncultured *Aeromonas* sp. clone ASP-21 16S ribosomal RNA gene, parital sequence	97%
SBS2-R13	EF198469	*Ralstonia* sp. BP2 16S ribosomal RNA gene, partial sequence	98%
SBS2-R14	U37342	*Burkholderia* sp. isolate N2P5 16S ribosomal RNA (rrn) gene, partial sequence	97%
SBS2-R15	AY136529	*Sphingomonas* sp. A-O20-1 16S ribosomal RNA gene, partial sequence	100%
SBS2-R16	AY512641	*Rhodococcus* sp. Amico42 16S ribosomal RNA gene, partial sequence	100%
SBS2-R17	AY580068	*Burkholderia* sp. 418 16S ribosomal RNA gene, partial sequence	98%
SBS2-R18	EF028124	*Rhodococcus* sp. Atl25 16S ribosomal RNA gene, partial sequence	98%
SBS2-R19	AB266612	*Cupriavidus* sp. KU-29 gene for 16S rRNA, partial sequence	98%
SBS2-R20	EF110914	*Arthrobacter* sp. CN-1 16S ribosomal RNA gene, partial sequence	100%
SBS2-R21	DQ196469	*Xanthomonas* sp. L60 16S ribosomal RNA gene, partial sequence	98%
SBS2-R22	AB091837	*Pseudomonas fluorescens* gene for 16S ribosomal RNA, partial sequence	100%
SBS2-R23	AB046362	*Rhodococcus erythropolis* gene for 16S rRNA, partial sequence	100%
SBS2-R24	AY921872	Uncultured *delta proteobacterium* clone AKYH836 16S ribosomal RNA gene, partial sequence	99%
SBS2-R25	DQ303434	*Pseudomonas* sp. J2 16S ribosomal RNA gene, partial sequence	100%
SBS2-R26	DQ130045	Uncultured *Rhizobiales* bacterium clone LPR22 16S ribosomal RNA gene, partial sequence	94%
SBS2-R27	AY800383	*Acinetobacter calcoaceticus* 16S ribosomal RNA gene, partial sequence	99%
SBS2-R28	EU013944	*Wautersia* sp. BFXJ-6 16S ribosomal RNA gene, partial sequence	100%
SBS2-R29	AB334770	*Rhodococcus erythropolis* gene for 16S ribosomal RNA, partial sequence	99%
SBS2-R30	AM259891	Uncultured *Flexibacter* sp. partial 16S rRNA gene, clone TAA-5-25	100%

Table 26. Taxonomic assignment of randomly selected cDNA clone sequences from the inoculated box reactor degrading NNT supplemented with atrazine.

Clones	Accession #	Identity	%Similarity
SBS3R-1	AB334527	*Pseudomonas* sp. MPU 101 gene for 16S ribosomal RNA, partial sequence	98%
SBS3R-2	DQ910431	*Pseudomonas* sp. G1012 16S ribosomal RNA gene, partial sequence	99%
SBS3R-3	EF530572	*Pseudomonas aeruginosa* 16S ribosomal RNA gene, partial sequence	98%
SBS3R-4	AF228366	*Pseudomonas fluorescens bv. G* 16S ribosomal RNA gene, partial sequence	97%
SBS3R-5	AM497794	*Rhodococcus koreensis* partial 16S rRNA gene, strain Sedi2	97%
SBS3R-6	AY238507	*Ralstonia* sp. 80 16S ribosomal RNA gene, partial sequence.	98%
SBS3R-7	AY238506	*Burkholderia* sp. 14 16S ribosomal RNA gene, partial sequence	98%
SBS3R-8	DQ125827	Uncultured bacterium clone AKAU4050 16S ribosomal RNA gene, partial sequence	96%
SBS3R-9	EF221777	*Pseudomonas* sp. tap-9 16S ribosomal RNA gene, partial sequence	100%
SBS3R-10	AM259579	*Serratia marcescens* partial 16S ribosomal RNA gene, isolate MF42	99%
SBS3R-11	DQ986324	*Burkholderia* sp. SJ98 16S ribosomal RNA gene, partial sequence	96%
SBS3R-12	DQ376584	*Sphingobium* sp. TP340-2 16S ribosomal RNA gene, partial sequence	99%
SBS3R-13	DQ123777	Uncultured soil bacterium clone PAH-Feed-43 16S ribosomal RNA gene, partial sequence	97%
SBS3R-14	DQ887520	*Ralstonia* sp. PNP11 16S ribosomal RNA gene, partial sequence	96%
SBS3R-15	DQ272471	*Rhodococcus* sp. ADC4 16S ribosomal RNA gene, partial sequence	99%
SBS3R-16	AF228366	*Pseudomonas fluorescens bv. G* 16S ribosomal RNA gene, partial sequence	97%
SBS3R-17	DQ659593	*Sphingomonas* sp. Zp1 16S ribosomal RNA gene, partial sequence	98%
SBS3R-18	DQ229101	*Klebsiella* sp. TNT2 16S ribosomal RNA gene, partial sequence	97%
SBS3R-19	AB167248	*Arthrobacter* sp. c138 gene for 16S rRNA, partial sequence.	95%
SBS3R-20	AB167236	*Nocardioides* sp. c103 gene for 16S rRNA, partial sequence	100%
SBS3R-21	AF439776	*Burkholderia* sp. JRB1 16S ribosomal RNA gene, partial sequence	96%
SBS3R-22	AY512641	*Rhodococcus sp. Amico42* 16S ribosomal RNA gene, partial sequence	96%
SBS3R-23	EF016828	Uncultured *actinobacterium* clone E1B-H7-114 16S ribosomal RNA gene, partial sequence	98%
SBS3R-24	D14016	*Flavobacterium balustinum* 16S ribosomal RNA	100%
SBS3R-25	AB241588	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone:d17	100%
SBS3R-26	EF362637	*Pseudomonas aeruginosa* strain 8 16S ribosomal RNA gene, partial sequence	98%
SBS3R-27	DQ417330	*Pseudomonas fluorescens* strain 3B 16S ribosomal RNA gene, partial sequence	99%
SBS3R-28	AY745846	*Micrococcus* sp. JL-76 16S ribosomal RNA gene, partial sequence	97%
SBS3R-29	AB245351	*Xanthobacteraceae* bacterium Gsoil 062 gene for 16S rRNA, partial sequence	98%
SBS3R-30	AB298540	*Agrobacterium* sp. BK22 gene for 16S rRNA, partial sequence	99%

Table 27. Taxonomic assignment of randomly selected cDNA clone sequences from the uninoculated box reactor degrading NNT supplemented with cyanuric acid.

Clones	Accession #	Identity	%Similarity
SBS4-R1	DQ912807	*Pseudomonas putida* strain ZX-PKU-004 16S ribosomal RNA gene, partial sequence	96%
SBS4-R2	EF392658	*Pseudomonas* sp. PD1 16S ribosomal RNA gene, partial sequence	99%
SBS4-R3	AB192294	*Bacillus subtilis* gene for 16S rRNA, partial sequence	97%
SBS4-R4	EF362636	*Rhodococcus erythropolis* strain 5 16S ribosomal RNA gene, partial sequence	98%
SBS4-R5	EU137140	*Pseudomonas* sp. P4-7 16S ribosomal RNA gene, partial sequence	97%
SBS4-R6	EU127939	Uncultured bacterium clone pla-1368-0.1m-53 16S ribosomal RNA gene, partial sequence	100%
SBS4-R7	AJ011508	*Burkholderia* sp. (strain DhA-54) 16S rRNA gene, partial sequence	98%
SBS4-R8	EU004416	*Rhodococcus globerulus* isolate AK36 16S ribosomal RNA gene, partial sequence	99%
SBS4-R9	AY771747	*Pseudomonas* sp. KOPRI 20902 16S ribosomal RNA gene, partial sequence	98%
SBS4-R10	AY226509	*Corynebacterium halophilum* 16S ribosomal RNA gene, partial sequence	100%
SBS4-R11	EF672104	*Pseudomonas* sp. B6-2 16S ribosomal RNA gene, partial sequence	99%
SBS4-R12	AM402975	*Mycobacterium* sp. 6PY1 partial 16S rRNA gene, strain 6PY1	98%
SBS4-R13	DQ676344	Uncultured *alpha proteobacterium* clone MVP-101 16S ribosomal RNA gene, partial sequence	96%
SBS4-R14	AY714238	*Pseudomonas putida* 16S ribosomal RNA gene, partial sequence	97%
SBS4-R15	EF530572	*Pseudomonas aeruginosa* 16S ribosomal RNA gene, partial sequence	98%
SBS4-R16	AJ630271	Uncultured *Flavobacterium* sp. partial 16S rRNA gene, clone MFC-EB1	98%
SBS4-R17	DQ011232	*Rhodococcus* sp. YU6 16S ribosomal RNA gene, partial sequence	99%
SBS4-R18	AY714235	*Arthrobacter* sp. HSL-2 16S ribosomal RNA gene, partial sequence	97%
SBS4-R19	AY771747	*Pseudomonas* sp. KOPRI 20902 16S ribosomal RNA gene, partial sequence	98%
SBS4-R20	EU124556	*Bacillus subtilis* strain OSS 5 16S ribosomal RNA gene, partial sequence	99%
SBS4-R21	EF494232	*Streptomyces* sp. CS38 16S ribosomal RNA gene, partial sequence	98%
SBS4-R22	DQ272470	*Pseudomonas* sp. ADC3 16S ribosomal RNA gene, partial sequence	97%
SBS4-R23	EF990634	*Burkholderia cepacia* strain PCL3 16S ribosomal RNA gene	98%
SBS4-R24	AY785735	*Rhodococcus* sp. OUCZ35 16S ribosomal RNA gene, partial sequence	97%
SBS4-R25	EF221772	*Stenotrophomonas* sp. tap-1 16S ribosomal RNA gene, partial sequence	99%
SBS4-R26	DQ399760	*Brevibacterium* sp. B-5131 16S ribosomal RNA gene, partial sequence	99%
SBS4-R27	EU137140	*Pseudomonas* sp. P4-7 16S ribosomal RNA gene, partial sequence	97%
SBS4-R28	AY741160	*Sphingomonas* sp. S31 16S ribosomal RNA gene, partial sequence	98%
SBS4-R29	EU124561	*Rhodococcus* sp. strain OSS 26 16S ribosomal RNA gene, partial sequence	96%
SBS4-R30	AY714238	*Pseudomonas putida* 16S ribosomal RNA gene, partial sequence	98%

Table 28. Taxonomic assignment of randomly selected cDNA clone sequences from the uninoculated box reactor degrading NNT supplemented with melamine.

Clones	Accession #	Identity	%Similarity
SBS5-R1	AJ011508	*Burkholderia* sp. (strain DhA-54) 16S rRNA gene, partial	98%
SBS5-R2	EF515832	*Pseudomonas aeruginosa* strain PT121 16S ribosomal RNA gene	100%
SBS5-R3	DQ229101	*Klebsiella* sp. TNT2 16S ribosomal RNA gene, partial sequence	97%
SBS5-R4	AB245351	*Xanthobacteraceae* bacterium Gsoil 062 gene for 16S rRNA, partial sequence	98%
SBS5-R5	EU100397	*Acinetobacter* sp. CP-B 16S ribosomal RNA gene, partial sequence	96%
SBS5-R6	AB188781	Uncultured *delta proteobacterium* gene for 16S rRNA, partial sequence	99%
SBS5-R7	EF028124	*Rhodococcus* sp. Atl25 16S ribosomal RNA gene, partial sequence	98%
SBS5-R8	EU017404	*Rhodococcus* sp. BFXJ-2 16S ribosomal RNA gene, partial sequence	98%
SBS5-R9	EF530572	*Pseudomonas aeruginosa* 16S ribosomal RNA gene, partial sequence	98%
SBS5-R10	EF640968	*Bacillus* sp. NAP2-2 16S ribosomal RNA gene, partial sequence	98%
SBS5-R11	DQ659593	*Sphingomonas* sp. Zp1 16S ribosomal RNA gene, partial sequence	98%
SBS5-R12	AB087725	*Nocardioides* sp. H-2 gene for 16S rRNA, partial sequence	98%
SBS5-R13	EU124561	*Rhodococcus* sp. strain OSS 26 16S ribosomal RNA gene, partial sequence	99%
SBS5-R14	AB096215	*Gamma proteobacterium* Y-134 gene for 16S rRNA, partial sequence	98%
SBS5-R15	AY914072	*Corynebacterium* sp. R-4 16S ribosomal RNA gene, partial sequence	100%
SBS5-R15	AF006504	Unidentified *pseudomonad* ps.15 16S ribosomal RNA gene, partial sequence	98%
SBS5-R16	AJ245702	*Mycobacterium* sp. partial 16S rRNA gene, strain LB501T	98%
SBS5-R17	AY651317	*Arthrobacter* sp. AG1 16S ribosomal RNA gene, partial sequence	97%
SBS5-R18	DQ858963	*Rhodococcus ruber* strain Z57 16S ribosomal RNA gene, partial	97%
SBS5-R20	AF448032	*Burkholderia* sp. S1-17 16S ribosomal RNA gene, partial sequence	97%
SBS5-R21	AY623816	*Pseudomonas oleovorans* 16S ribosomal RNA gene, partial sequence	96%
SBS5-R22	EU017404	*Rhodococcus* sp. BFXJ-2 16S ribosomal RNA gene, partial sequence	97%
SBS5-R23	AB066236	*Flavobacterium* sp. PJ711 gene for 16S ribosomal RNA, partial sequence	96%
SBS5-R24	DQ912807	*Pseudomonas putida* strain ZX-PKU-004 16S ribosomal RNA gene, partial sequence	99%
SBS5-R25	EF577242	*Streptomyces* sp. S096 16S ribosomal RNA gene, partial sequence	98%
SBS5-R26	AY771802	*Sphingomonas* sp. Gamma1-7 16S ribosomal RNA gene, partial sequence	96%
SBS5-R27	EF530572	*Pseudomonas aeruginosa* 16S ribosomal RNA gene, partial sequence	97%
SBS5-R28	AB334770	*Rhodococcus erythropolis* gene for 16S ribosomal RNA, partial sequence	98%
SBS5-R29	EU017404	*Rhodococcus* sp. BFXJ-2 16S ribosomal RNA gene, partial sequence	97%
SBS5-R30	EF645802	*Serratia marcescens* strain 65-FAR1 16S ribosomal RNA gene, partial sequence	97%

Table 29. Taxonomic assignment of randomly selected cDNA clone sequences from the uninoculated box reactor degrading NNT supplemented with atrazine.

Clones	Accession #	Identity	%Similarity
SBS6-R1	EU037096	*Pseudomonas aeruginosa* strain CMG860 16S ribosomal RNA gene	98%
SBS6-R2	DQ173027	*Arthrobacter* sp. TSBY-75 16S ribosomal RNA gene, partial sequence	97%
SBS6-R3	EF694073	*Pseudomonas fluorescens* strain yl 16S ribosomal RNA gene, partial sequence	98%
SBS6-R4	EF450777	*Rhodococcus* sp. P3 16S ribosomal RNA gene, partial sequence	99%
SBS6-R5	AB007995	*Ralstonia eutropha* gene for 16S rRNA, partial sequence	97%
SBS6-R6	DQ912807	Pseudomonas putida strain ZX-PKU-004 16S ribosomal RNA gene, partial sequence	97%
SBS6-R7	AB334770	*Rhodococcus erythropolis* gene for 16S ribosomal RNA, partial sequence	99%
SBS6-R8	AY345415	Bacterium K2-47 16S ribosomal RNA gene, partial sequence	97%
SBS6-R9	EF990557	*Bacillus subtilis* strain DA1 16S ribosomal RNA gene, partial sequence	98%
SBS6-R10	AB012207	*Corynebacterium* sp. gene for 16S rRNA, partial sequence	99%
SBS6-R11	DQ882847	*Streptomyces* sp. 10-4 16S ribosomal RNA gene, partial sequence	98%
SBS6-R12	AM412170	*Flavobacterium* sp. P-139 partial 16S rRNA gene, strain P-139	96%
SBS6-R13	DQ279760	*Pseudomonas* sp. L54 16S ribosomal RNA gene, partial sequence	97%
SBS6-R14	EF509593	Uncultured bacterium clone P7D82-685 16S ribosomal RNA gene, partial sequence	98%
SBS6-R15	AF005994	*Pseudomonas* sp. 679-2 16S ribosomal RNA gene, partial sequence	98%
SBS6-R15	DQ912807	*Pseudomonas putida* strain ZX-PKU-004 16S ribosomal RNA gene, partial sequence	97%
SBS6-R16	AY238506	*Burkholderia* sp. 14 16S ribosomal RNA gene, partial sequence	98%
SBS6-R17	EU046269	*Flavobacterium anhuiense* strain D3 16S ribosomal RNA gene, partial sequence	95%
SBS6-R18	AY913408	Uncultured forest soil bacterium clone DUNssu202 16S ribosomal RNA	96%
SBS6-R20	AJ009706	*Sphingomonas* sp. K101 partial 16S rRNA gene, isolate K101	98%
SBS6-R21	DQ001072	*Rhodococcus* sp. F 16S ribosomal RNA gene, partial sequence	96%
SBS6-R22	EF694073	*Pseudomonas fluorescens* strain yl 16S ribosomal RNA gene, partial sequence	97%
SBS6-R23	DQ659593	*Sphingomonas* sp. Zp1 16S ribosomal RNA gene, partial sequence	98%
SBS6-R24	AB007995	*Ralstonia eutropha* gene for 16S rRNA, partial sequence	97%
SBS6-R25	AY714235	*Arthrobacter* sp. HSL-2 16S ribosomal RNA gene, partial sequence	96%
SBS6-R26	DQ887520	*Ralstonia* sp. PNP11 16S ribosomal RNA gene, partial sequence	97%
SBS6-R27	EU037096	*Pseudomonas aeruginosa* strain CMG860 16S ribosomal RNA gene	98%
SBS6-R28	AB266607	*Burkholderia* sp. KU-25 gene for 16S rRNA, partial sequence	98%
SBS6-R29	EF538720	*Nocardia* sp. 171103 16S ribosomal RNA gene, partial sequence	96%
SBS6-R30	AF447392	*Rhodococcus aetherovorans* strain Bc663 16S ribosomal RNA gene	97%

Growth of Isolates from Activated Sludge and Woodchips that Degrade High Concentrations of NNT Supplemented with either Cyanuric Acid, Melamine, or Atrazine

Organisms isolated from sludge and box reactors were evaluated on how well they grew on increasing concentrations of NNT supplemented with cyanuric acid, melamine, or atrazine. Metabolism of compounds was measured by growth of isolates on Stanier's plates that had been incubated for 1 week at 30^{o}C (Plates were done in triplicates). Tables 30-32 show that some organisms prefer one nitrogen source over another when degrading elevated levels of NNT. Isolate A showed excellent to good growth with elevated concentrations of NNT using cyanuric acid, excellent to marginal growth using melamine, and good growth to little growth using atrazine. Isolate B showed very good to good growth with increasing concentrations of NNT using cyanuric acid, excellent to good growth using melamine and good to no growth using atrazine. Isolate C displayed excellent to good growth using cyanuric acid, and good to little growth in the presence of melamine and atrazine. Isolate D showed excellent to good growth in the presence of cyanuric acid and melamine with increasing concentration of NNT, and very good to good growth using atrazine as a nitrogen source. Isolate E showed good growth on all concentrations of NNT using cyanuric acid, excellent to good growth using melamine, and excellent to no growth with atrazine. Isolate F showed very good growth to little growth using cyanuric acid and melamine, and good growth to little growth using atrazine. Isolate G showed excellent growth to very good growth in the presence of cyanuric acid, excellent to good growth when using melamine and atrazine. Isolate H showed excellent to little growth in conditions with cyanuric acid and melamine, and excellent to no growth using atrazine. Isolate I showed excellent growth to good growth with cyanuric acid, excellent to very good growth using melamine as nitrogen source and very good growth to good growth in the presence of atrazine. Isolate J showed excellent to little growth with cyanuric acid and very good growth to good

growth using melamine and atrazine. Isolates obtained from woodchips and activated sludge metabolize high concentrations of NNT in the presence of either cyanuric acid, melamine, or atrazine. However, the ranges of their growth vary depending on which nitrogen source is used and the concentrations of NNT that they must metabolize.

Table 30. Growth of isolates A-J on increasing concentrations of both NNT and cyanuric acid.

Isolates	**NNT1**	**NNT2**	**NNT3**	**NNT4**
A	++++	++++	++	+++
B	++	+++	++	++
C	++++	++	+++	++++
D	+++	++++	++	++
E	++	++	++	++
F	++	++	+++	+
G	+++	++++	+++	++++
H	++++	+	+	++
I	++++	++++	++	+++
J	++++	++	+	++

NNT1= 25ppm of Cyanuric Acid, 250 ppm of each NT and 50ppm of NoBz

NNT2= 50ppm of Cyanuric Acid, 500 ppm of each NT and 100ppm of NoBz

NNT3= 75 ppm of Cyanuric Acid, 750 ppm of each NT and 125ppm of NoBz

NNT4=100ppm of Cyanuric Acid, 1000ppm of each NT and 150ppm of NoBz

-No detectable growth

+/- Marginal growth

\+ Little growth

++ Good growth

+++ Very good growth

++++ Excellent growth

Table 31 . Growth of isolates A-J on increasing concentrations of both NNT and melamine.

Isolates	NNT1	NNT2	NNT3	NNT4
A	+ + +	+ + + +	++	+/-
B	+ + +	+ + +	+ + + +	+ +
C	+ +	+	+ +	+ +
D	+ + + +	+ + + +	+ + + +	+ +
E	+ +	+ + + +	+ +	+ + +
F	+	+ +	+ +	+ + +
G	+ + + +	+ + + +	+ + + +	+ +
H	+ + + +	+ + +	+	+ +
I	+ + +	+ + +	+ + + +	+ + +
J	+ +	+ +	+ + +	+ +

NNT1= 25ppm of Melamine, 250 ppm of each NT and 50ppm of NoBz

NNT2= 50ppm of Melamine, 500 ppm of each NT and 100ppm of NoBz

NNT3= 75 ppm of Melamine ,750 ppm of each NT and 125ppm of NoBz

NNT4=100ppm of Melamine , 1000 ppm of each NT and 150ppm of NoBz

-No detectable growth

+/- Marginal growth

\+ Little growth

++ Good growth

+++ Very good growth

++++ Excellent growth

Table 32. Growth of isolates A-J on increasing concentrations of both NNT and atrazine.

Isolates	NNT1	NNT2	NNT3	NNT4
A	+ +	+	+	+
B	+ +	+ +	+ /-	-
C	+ +	+	+ +	+
D	+ +	+ + +	+ +	+ +
E	+ + +	+ + + +	+ +	+
F	+	+	+ +	+
G	+ + +	+ + + +	+ +	+ +
H	+ + + +	+	+	-
I	+ + +	+ + +	+ +	+ + +
J	+ +	+ +	+ + +	+ +

NNT1= 25ppm of Atrazine, 250 ppm of each NT 50ppm of NoBz

NNT2= 50ppm of Atrazine, 500 ppm of each NT 100ppm of NoBz

NNT3= 750ppm of Atrazine, 750 ppm of each NT 125ppm of NoBz

NNT4=100ppm of Atrazine, 1000 ppm of each NT and 150ppm of NoBz

-No detectable growth

+/- Marginal growth

\+ Little growth

++ Good growth

+++ Very good growth

++++ Excellent growth

Chemotaxis

The chemotactic response of isolates A-J to NNT using cyanuric acid, melamine, and atrazine were analyzed on Stanier's swarm plates. Isolates A-J could be readily distinguished by their chemotactic behavior on Stanier's swarm plates. Strains A-J responded chemotactically to a variety of compounds in an assay in which the gradient of attractant, NNT, is generated by consumption, suggesting that chemotaxis might be a major mechanism to help this microorganism to find an environment that supports optimal growth.

Pure cultures isolated from woodchips and activated sludge were tested using the swarm plate chemotaxis assay to see how attracted they were to NNT using cyanuric acid, melamine, and atrazine as a nitrogen source. Isolate A was more attracted to NNT using cyanuric acid than in the conditions with melamine and atrazine. Isolate B was one of the poorer chemotactic organisms with less than 1.0 cm of growth in the presence of cyanuric acid, melamine, and atrazine. Isolate C was more attracted to NNT in the presence of cyanuric acid and melamine as opposed to atrazine. Isolate D showed more attraction to NNT using melamine as a nitrogen source as opposed to using cyanuric acid and atrazine, in which the chemotactic response was not as strong. Isolate E exhibited more attraction to VOCs in the presence of cyanuric acid and melamine than in atrazine. Isolate F showed the least chemotactic response to NNT when using the nitrogen sources being studied. Isolate G showed excellent chemotactic response to NNT using the experimental nitrogen sources. Isolate H and I were more attracted to NNT in the presence of cyanuric acid as opposed to conditions where melamine and atrazine were present.

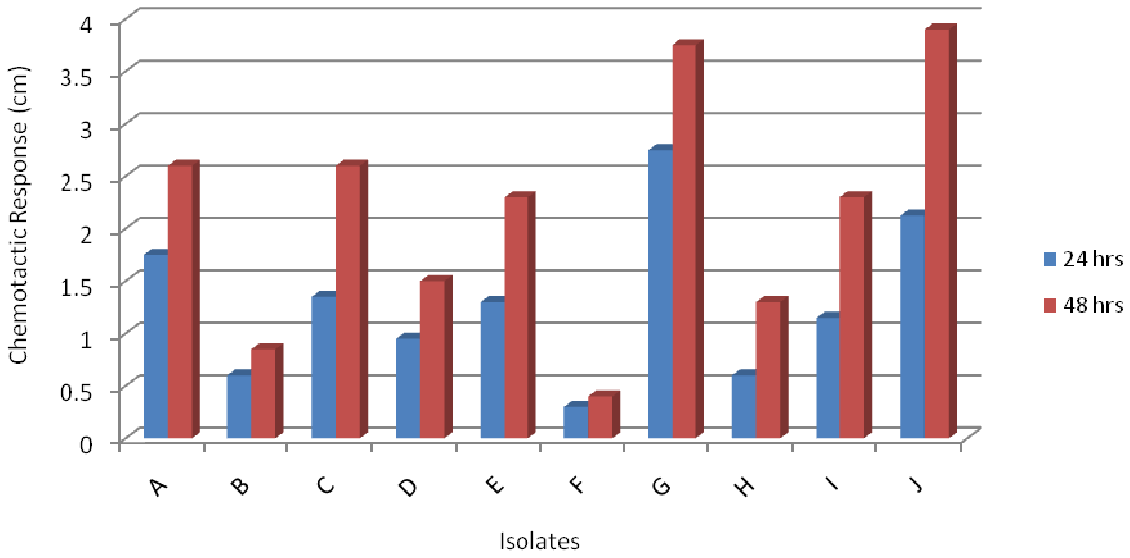

Figure 38. Chemotactic response towards NNT supplemented with cyanuric acid.

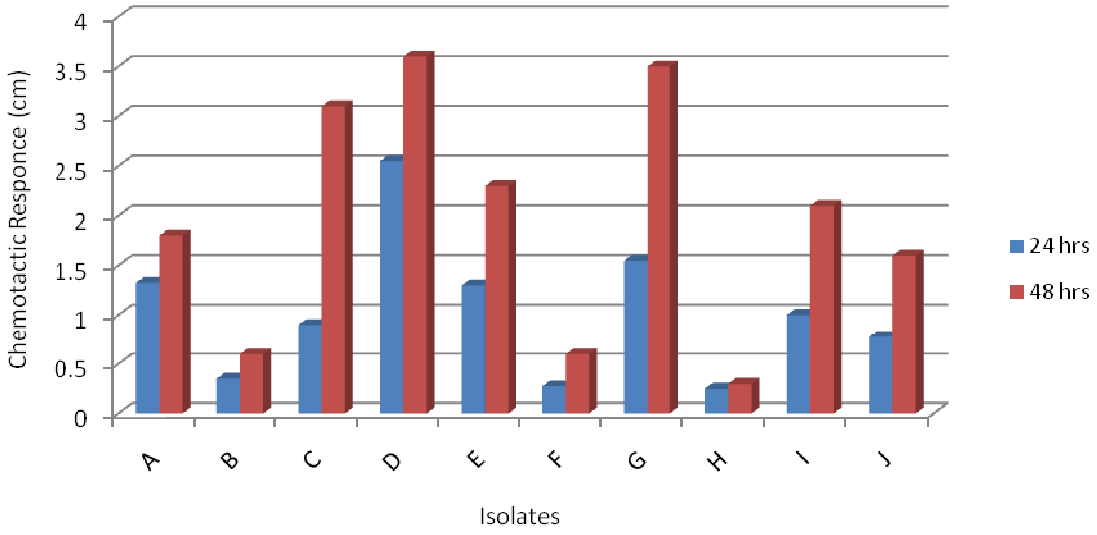

Figure 39. Chemotactic response towards NNT supplemented with melamine.

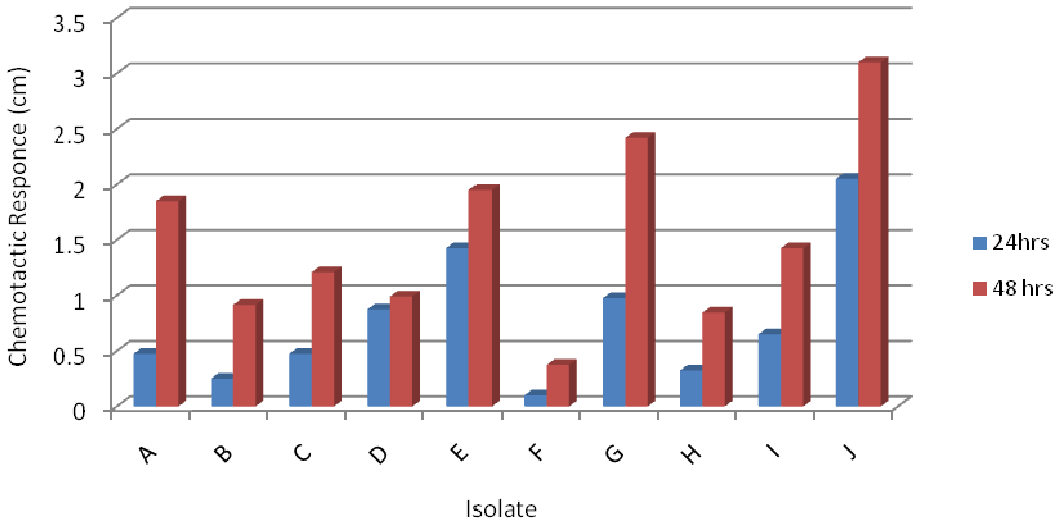

Figure 40. Chemotactic response towards NNT supplemented with atrazine.

Identification of Isolates from Activated Sludge and Woodchips that Degrade High Concentrations of NNT Supplemented with either Cyanuric Acid, Melamine, or Atrazine

The bacterial species were isolated from woodchips and activated sludge by the spread plate technique. Isolated colonies were serially transferred to Stainer's supplemented with NNT. This type of media only selected for specific hydrocarbon degraders. After successive transfers to varying concentrations of NNT, in the presence of cyanuric acid, melamine, and atrazine, the colonies were characterized by morphology and identified by analysis of the sequence of the gene encoding 16S rRNA. Universal primers 338F and 907 R were used to amplify partial regions of the 16S rRNA gene. The characterization of the bacterial isolates obtained revealed the following genera: *Arthrobacter*, *Pseudomonas*, *Rhodococcus*, *Ralstonia*, *Oligotropha*, *Comanous, Zoogloea*, *and Sphaerobacte*r. All of these isolates were able to grow on NNT as sole source of carbon and energy,

Table 33. Identification of Isolated cultures from woodchips and sludge that can degrade high concentration of NNT.

Isolates	Morphological Characteristics	Acession #	Identity	%Similarity
A	Cream colored colonies	AY336115	*Arthrobacter woluwensis* strain PSD-5 16S ribosomal RNA gene, partial sequence	100%
B	Circular, brown center, concave	DQ981491	*Pseudomonas* sp. MG-4 16S ribosomal RNA gene, partial sequence	97%
C	Rough orange colonies	AF501338	*Rhodococcus erythropolis* strain HAMBI2393 16S ribosomal RNA gene, partial sequence	95%
D	Cream colored colonies	AB164432	*Comamonas badia* gene for 16S rRNA, partial sequence	97%
E	White large smooth colonies	AF225956	*Pseudomonas aeruginosa* 16S ribosomal RNA gene, partial sequence	97%
F	Grey rough colonies	AB099660	*Oligotropha carboxidovorans* gene for 16S rRNA, complete sequence, strain: S28	100%
G	Tan muccoid colonies	DQ342276	*Zoogloea* sp. A5 16S ribosomal RNA gene, partial sequence	97%
H	Brownish green small smooth colonies	X53210	*Sphaerobacter thermophilus* (Corynebacterium spec.) partial 16S r RNA	100%
I	Circular, small rough white colonies	DQ990336	*Pseudomonas* sp. PYX1 16S ribosomal RNA gene, partial sequence	100%
J	Yellowish green large smooth colonies	DQ997838	*Ralstonia pickettii* strain HM-1 16S ribosomal RNA gene, partial sequence	97%

IV. DISSCUSION

Contamination of soils from petroleum hydrocarbons by leaking underground storage tanks and various industrial operations are a continuous threat to the environment. Currently there are many clean up methods available. However, bioremediation has gained approval based on its removal efficiency and cost effectiveness when compared to other technologies such as incineration and land-farming (Lageman *et al*., 2005). The remediation of hazardous wastes such as NNT by non-biological process is estimated to cost the US $750 billion over the next 30 years, whereas bioremediation is significantly lower, at $75 billion (Pimentel *et. al*, 1997).

Nitrogen is often a limiting factor in heavily polluted environments. The addition of inexpensive slow release nitrogen sources have been successfully used in the degradation of petroleum hydrocarbons (Lee *et al*., 1995). Cyanuric acid, melamine, and atrazine are slow release nitrogen sources that can be used as the sole nitrogen source for growth for *Pseudomonas* strains (Cook and Huttner, 1981). Similarly, these compounds were used as supplemental nitrogen sources for microbial communities within box reactors during the degradation of NNT.

Solid phase bioremediation was used within this dissertation research in the degradation of NNT supplemented with cyanuric acid, melamine, or atrazine. The microbial breakdown of NNT within box reactors included stimulation of the native microorganism by optimization of nutrients, oxygen, and through the inoculation of specific degrading microbial consortiums. Community analysis studies, followed by the isolation and identification of NNT degraders, were performed on degrading communities to get an idea of which organisms were actively participating in the degradation process.

Experimental box reactors were inoculated with DAP strains 66, 119, and 622. These strains demonstrated acclimation, growth and extended survival times on Stanier's plates with

increasing concentrations of NNT supplemented with either cyanuric acid, melamine, and atrazine (tables 2,3,4). Bioremediation studies have shown that the introduction of microorganisms enhances the removal and rate of removal of compounds, and that the indigenous populations appeared to be incompetent as compared to augmented strains (Brigliea, *et al*., 1994). Bioaugmentation has been more successful in treating recalcitrant compounds, such as PAHs, in comparison to soils that were not augmented (Rutherford *et. al*., 1998, and Juhasz *et al.*, 2000).

All strains were previously induced to withstand high concentrations of NNT and inoculated into the experimental box reactors at a concentration of 10^6/g soil. Effective bioaugmented bioremediation systems have shown that using high numbers of acclimated known degraders (>10^6/g soil) in contaminated soil environments resulted in targeted compounds being efficiently degraded (Bossert and Bartha, 1984).

Box reactors shown in figures 2, 4, and 8 represent environments that had not been exposed to high concentrations of NNT. Degradation within these boxes took three weeks. The slow degradation of NNT was a result of acclimation by the microbial communities within box reactors to NNT with supplemental nitrogen sources. During this acclimation period, the appropriate degrading microbial communities were selected for through growth and by enzyme induction.

The degradation of increasing concentrations of NNT supplemented with either cyanuric acid, melamine, or atrazine after two applications of NNT is illustrated in figures 2, 5, 8, 11, 14, 17, 20, 22, 26, 28, 32, and 35. Rates of degradation within all the tables show that toluene is degraded the fastest followed by naphthalene and nitrobenzene (tables 5-16). The degradation rate not only includes microbial degradation but volatilization of a compound. The rate of

toluene removal from all environments is faster than naphthalene because toluene is being volatized more quickly than naphthalene and nitrobenzene. Higher degradation rates are seen as concentrations increase to 3000ppm of NT and 300 NoBz, made evident by the fact that NNT is still being degraded within the same period of time as lower concentrations. The degradation within box reactors supplemented with cyanuric acid, melamine, and atrazine improves over time because they have previously been exposed to high concentrations of NNT. The overall degradation rates of NNT in experimental box reactors were similar to control box reactors. Differences were seen, but they were not great enough to say that inoculated box reactors degraded NNT faster than control box reactors. This was attributed to the fact that communities within both the experimental and control box reactors were established due to the continuous application of NNT and supplemental nitrogen sources. The outcome of bioaugmentation within any bioremediation system is unpredictable due to the variation of strains that may be selected for in a particular environment. NNT all have different sizes and solubility's and therefore were expected to degrade differently within each of the given environments. Eriksson *et al.*, 2000 saw that there was no difference in degradation of naphthalene and phenanthrene in inoculated and uninoculated soil microcosms. Degradation within all soil microcosms was achieved within 20 days to either low or non-detectable levels with continuous aeration and addition of water.

Box reactors contained VOC traps in order to account for losses due to volatilization so as to not overestimate the extent of NNT bioremediation. At the lowest concentrations of NNT supplemented with test nitrogen sources, more than half of toluene was lost to volatilization, not microbial degradation. Less than 15% of naphthalene was removed as a result of volatilization. Volatilization of nitrobenzene fluctuated between 5-25% in box reactors, with the highest rate seen in the atrazine control box (figures 3,6,9).

Differences in losses associated with volatilization were seen at concentrations of 1000ppm NT and 100ppm of NoBz supplemented with cyanuric acid, melamine, or atrazine. Figures 12, 14, and 18 illustrate that toluene in experimental and control box reactors supplemented with cyanuric acid and melamine showed less than 40% of removal was due to volatilization ,where as toluene loss in the atrazine box reactors was greater than 50% in the control box, and less than 40% in the experimental box reactor. The amount of naphthalene lost to volatilization was less than 10% in the experimental and control box reactors supplemented with cyanuric acid and melamine, and less than 15% in box reactors supplemented with atrazine. Less than 10% of NoBz volatized in box reactors supplemented with melamine. An average volatilization loss of 10% was seen in box reactors supplemented with cyanuric acid and atrazine.

At a concentration of 2000ppm of NT and 200ppm of NoBz in box reactor supplemented with cyanuric acid, melamine, or atrazine, the percentage of compounds volatized was similar to that seen at 1000ppm of NT and 100ppm of NoBz. Toluene in experimental and control box reactors volatized in a range between 30-40%, and naphthalene and nitrobenzene were lost on average of 10%. Similarly, box reactors containing 3000ppm of NT and 300 ppm of NoBz supplemented with cyanuric acid, melamine, or atrazine showed toluene volatilization to be between 30-40%. Naphthalene and nitrobenzene showed volatilization losses of less than 10% in reactors supplemented with cyanuric acid and melamine, and about a 10% loss in the box reactor supplemented with atrazine.

The majority of NNT volatilized within the first 4 days of the degradation runs. Toluene was the most volatile compound because its sorption to organic matter is weaker than naphthalene and nitrobenzene. The volatilization rates seen within box reactors, at the

beginning of the degradation runs, suggest that microbial communities were in the early stages of adapting to NNT using additional nitrogen sources, which resulted in less degradation attributed to microorganisms. As higher concentrations of NNT were placed in the experimental and control box reactors, volatilization losses decreased. The organisms within experimental and control boxes were optimizing their nitrogen usage, thus degrading the compounds faster and more efficiently. Salanitro (2001) saw that 60% of fuel hydrocarbons were lost during soil bioremediation primarily due to volatilization. Hawthorne and Grabnski (2000) showed that during the treatment of contaminated soils, semi-volatile PAHs volatized greater than 30%.

When the box reactors were started, the time to remediate low concentrations of NNT was three weeks (figures 4, 7, and 10). Before the refeed, NNT was totally removed from box reactors supplemented with cyanuric acid and all compounds, except naphthalene, in the control box was removed from box reactors containing atrazine. Box reactors supplemented with melamine showed that 20% of nitrobenzene remained along with 2% of naphthalene in box reactors. After the refeed, all box reactors supplemented with cyanuric acid, melamine, and atrazine were remediated. The removal differences seen before and after the reefed in experimental and control box reactors supplemented with melamine suggests that the supplemented nitrogen sources influenced the removal of NNT.

As the concentrations in experimental and control boxes were increased to 1000, 2000, and 3000 ppm of NT and 100, 200, and 300 ppm of NoBz, a similar trend to that of NNT remediation at the lowest concentrations were observed (figures13, 16, 19, 22, 25, 28, 31, 34, 37). After the reefed, 95% or more of NNT remediation was achieved in experimental and control box reactors. However, before the refeed, the nitrogen source that was supplemented within each of the box reactors may have influenced how much of NNT was removed. For

example, a comparison of figures 13, 16, and 19 shows that NNT was not completely remediated in box reactors supplemented with atrazine. Naphthalene and nitrobenzene persisted in box reactors supplemented with cyanuric acid, and naphthalene persisted in box reactors supplemented with melamine. However, it must be noted that significant removal was still achieved in all box reactors before the reefed. As concentrations of NNT increased, naphthalene appeared to be the most recalcitrant compound within the environments, followed by nitrobenzene. Naphthalene and NoBz were not completely remediated in soil boxes because they had sorbed to the organic matter making them less bioavailable to biodegrading microorganisms.

Many studies have described PAH contaminated environments through the use of 16s rRNA analysis, however little research has been done on microbial communities using slow release nitrogen sources to degrade aromatics and PAHs. There are many factors which influence the structure of a microbial community, but the main factors that influenced communities within box reactors was the selection pressure imparted by NNT supplemented with cyanuric acid, melamine, or atrazine, and the cell surface carrier (woodchips). To study the bacterial community and track the survival of inoculated strains, a combined use of 16s rDNA and 16s rRNA was performed. 16s rDNA analysis was done in order to investigate the microbial diversity within each box reactor, whereas 16s rRNA analysis was used to identify metabolically active microorganisms present in degrading communities.

Inoculated box reactors contained a mixed culture consisting of DAP strains 66, 119, and 622. These strains were identified by 16s rDNA and were most similar to *Rhodococcus amico* 42, *Aeromonas* sp. PW1 and *Pseudomonas fluorescens* (table 17). Theses sequences were compared to the cloned sequences from inoculated boxes using both 16s rDNA and 16s rRNA

(tables 18-29). In inoculated box reactors, only *Rhodococcus amico* 42, and *Pseudomonas fluorescens* were present within the clone libraries. This implies that not only were these organisms present within the environments, but that they were also active during degradation of NNT. The survival of these strains within environments is not surprising. They both were isolated from soil near Bridgewater, NJ (Pierce and Smith, 1997). *Aeromonas* sp. PW1 was not present within clone libraries. The conditions within the soil box selected for populations that were best adapted to environmental conditions in the presence of NNT. The *Aeromonas* sp. might be present within the environment, however its absence from clone libraries suggests that it exist as a minority within the community or has been selected against. Degrading strains can be poor survivors or lose catabolic activity in inoculation into non-sterile soils (Cavalca,2002).

Clone libraries obtained from 16s rDNA and 16s rRNA from box reactors both show the dominance of proteobacteria and gram positive bacteria. Certain genera were more prevalent in the 16s rRNA clone libraries as oppose to 16s rDNA libraries, such as *Rhodococcus*, *Pseudomonas*, *Burkholderia*, *Arthrobacter*, and *Sphingomonas*. These organisms have been seen in other clone libraries from community studies done in polluted environments (Nogales *et al*., 2001; Hamamura *et al*., 2006; Janssen, 2006). Analysis of PCR-amplified sequences received from the box reactors are not quantitative, they are qualitative. However, the appearance of sequences related to DAP strains, and other known degraders, from the box reactors degrading NNT, suggest that the microorganisms represented by these sequences may play a significant role in the degradation of NNT. This information can be utilized to establish conditions within future bioremediation systems that will be optimized for organisms that are responsible for the degradation of NNT, ultimately enhancing bioremediation.

Ten NNT degrading cultures were isolated from woodchips pre-exposed to high concentrations of NNT and activated sludge. Bacterial isolates A-J, which were identified by 16s rDNA (table 33), belonged to the following genera: *Arthrobacter*, *Pseudomonas*, *Rhodococcus*, *Ralstonia*, *Oligotropha*, *Comanous, Zoogloea*, and *Sphaerobacte*r. The growth of isolates A-J on increasing concentrations of NNT supplemented with cyanuric acid, melamine, or atrazine was evaluated (table 30-32). All isolates could grow on NNT, however their growth varied as a result of which additional nitrogen source was applied. Isolates A- J were evaluated on their chemotactic response to NNT supplemented with either of the s-triazines on Stanier's swarm plates (figures 38-40). Similar to what was seen in growth tables, isolates had varying chemotactic responses to NNT. Since isolates A-J can grow on high concentration of NNT, and have some taxis towards compounds, they may be potential candidates for future bioaugmentation studies. Growth tables and chemotaxis results may be an indication of how isolates A-J might degrade hydrocarbons in either soil or in liquid environments.

The results received from this study can aid in the development of new bioremediation technologies that will reduce the time usually required for bioremediation of aromatics and PAHs. Within any biodegradation system, limiting factors will exist. However, these factors can be overcome by improving growth requirements for native microorganisms (aerations, moisture), isolating and identifying possible degraders, and overcoming contaminant bioavailability.

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